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PATENT TRADEMARK OFFICE

CHAPTER II

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

INTERNATIONAL APPLICATION NO. CLAIMED	INTERNATIONAL FILING DATE	PRIORITY DATE
PCT/NL99/00453	15 JULY 1999	16 JULY 1998 14 DECEMBER 1998

TITLE OF INVENTION
PROCESS TO COLLECT METABOLITES FROM MODIFIED NECTAR BY INSECTS

APPLICANT(S)

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Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date January 16, 2001, in an envelope as "Express Mail Post Office to Addressee," Mailing Label Number EL 728210604 US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

JENNIFER RASHKIN
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

****WARNING:*** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b)*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442*

NOTE *The completion of those filing requirements that can be made at a time later than 30 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 USC 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase See 37 C F R §1.491 which states: "An international application enters the national state when the applicant has filed the documents and fees required by 35 USC 371(c) within the periods set forth in § 1.494 and § 1.495."*

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C F R. §1 10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C.F.R. §1.8.*

NOTE: *Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F R § 1 494(f).*

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:

   a. [X] This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).

   b. [X] The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2.Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
[ ]*	TOTAL CLAIMS	30 - 20 =	10	x $ 18.00 =	$180.00 (NOT PAID AT THIS TIME)
	INDEPENDENT CLAIMS	7 - 3 =	4	x $ 80.00 =	320.00 (NOT PAID AT THIS TIME)
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $270.00				
BASIC FEE**	[ ] U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: [ ] and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) ..... $100.00 [ ] and the above requirements are not met (37 CFR 1.492(a)(1)) ..... $690.00 [X] U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: [ ] has been paid (37 CFR 1.492(a)(2)) ..... $710.00 [ ] has not been paid (37 CFR 1.492(a)(3)) ..... $1,000.00 [X] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) $860.00				
	Total of above Calculations				=860.00
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR 1.9, 1.27, 1.28)				-
	Subtotal				860.00
	Total National Fee				$860.00
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				
TOTAL	Total Fees enclosed				$860.00

*See attached Preliminary Amendment Reducing the Number of Claims.

i. [X] A check in the amount of 860.00 to cover the above fees is enclosed.
ii. [ ] Please charge Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

***WARNING:* *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date. * * * (2) the basic national fee (see § 1 492(a)). The 30-month time limit may not be extended " 37 C F R § 1 495(b)*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office 37 C F R § 1.495(b)(2). The payment of the surcharge set forth in § 1 492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1 492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date Failure to comply with these requirements will result in abandonment of the application The provisions of § 1.136 apply to the period which is set Notice of Jan 3, 1993, 1147 O.G. 29 to 40*

3. [X] A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE: *Section 1 495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20 At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47 1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36 See item 14c below*

a. [ ] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. [X] has been transmitted
i. [X] by the International Bureau.
Date of mailing of the application (from form PCT/IB/308): _____.
ii. [ ] by applicant on _____.
Date

4. [X] A translation of the International application into the English language (35 U.S.C. 371(c)(2)):
a. [X] is transmitted herewith.
b. [ ] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on _____.
Date
d. [ ] will follow.

5. [X] Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE: The Notice of January 7, 1993 points out that 37 C F R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that. "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments Applicant may submit that subject matter in a preliminary amendment filed under section 1 121 In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.

a. [ ] are transmitted herewith.
b. [ ] have been transmitted
   i. [ ] by the International Bureau.
   Date of mailing of the amendment (from form PCT/IB/308): _____.
   ii. [ ] by applicant on _____.
   Date
c. [X] have not been transmitted as
   i. [X] applicant chose not to make amendments under PCT Article 19.
   Date of mailing of Search Report (from form PCT/ISA/210): MAY 11, 1999.
   ii. [ ] the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):
a. [ ] is transmitted herewith.
b. [ ] is not required as the amendments were made in the English language.
c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [X] A copy of the international examination report (PCT/IPEA/409)
[X] is transmitted herewith.
[ ] is not required as the application was filed with the United States Receiving Office.

8. [X] Annex(es) to the international preliminary examination report
a. [X] is/are transmitted herewith.
b. [ ] is/are not required as the application was filed with the United States Receiving Office.

9. [X] A translation of the annexes to the international preliminary examination report
a. [X] is transmitted herewith.
b. [ ] is not required as the annexes are in the English language.

10. [X] An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115
    a. [ ] was previously submitted by applicant on _____. Date
    b. [ ] is submitted herewith, and such oath or declaration
        i. [ ] is attached to the application.
        ii. [ ] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.
    c. [X] will follow.

Other document(s) or information included:

11. [X] An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
    a. [X] is transmitted herewith.
    b. [ ] has been transmitted by the International Bureau.
    Date of mailing (from form PCT/IB/308): _____.
    c. [ ] is not required, as the application was searched by the United States International Searching Authority.
    d. [ ] will be transmitted promptly upon request.
    e. [ ] has been submitted by applicant on _____. Date

12. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
    a. [ ] is transmitted herewith.
    Also transmitted herewith is/are:
    [ ] Form PTO-1449 (PTO/SB/08A and 08B).
    [ ] Copies of citations listed.
    b. [X] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
    c. [ ] was previously submitted by applicant on _____. Date

13. [ ] An assignment document is transmitted herewith for recording.

A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [ ] FORM PTO 1595 is also attached.

_____
_____
_____

14. [X] Additional documents:
    a. [ ] Copy of request (PCT/RO/101)
    b. [X] International Publication No. WO /04176
        i. [X] Specification, claims and drawing
        ii. [ ] Front page only
    c. [ ] Preliminary amendment (37 C.F.R. § 1.121)
    d. [ ] Other

15. [X] The above checked items are being transmitted
    a. [X] before 30 months from any claimed priority date.
    b. [ ] after 30 months.

16. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1 17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C F R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1 26(a).

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 12-0425 .

[X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R § 1 495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

[ ] 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must

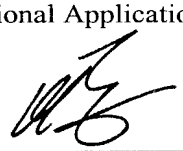
only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C F R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

[X] 37 C.F.R. 1.17 (application processing fees)
[X] 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).
[X] 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance 37 C F R § 1 311(b)

NOTE 37 C F R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . prior to paying, or at the time of paying . . issue fee." From the wording of 37 C.F.R. § 1.28(b) (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No.: 25,858

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NEW YORK, NEW YORK. 10023

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jantina CREEMERS, et al

Serial No.: 09/743,885 Group No.:

Filed: January 16, 2001 Examiner:

For: PROCESS TO COLLECT METABOLITES FROM MODIFIED NECTAR BY INSECTS

Attorney Docket No.: U-013212-4

Assistant Commissioner Patents and Trademarks
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above application as follows:

IN THE SPECIFICATION:

Please amend the paragraph beginning on page 19, line 21 as follows:

CERTIFICATE OF MAILING (37 CFR 1.8a)

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner of Patents and Trademarks, Washington, DC 20231

CLIFFORD J. MASS
Type or print name of person mailing paper)

Date: October 22, 2001

(Signature of person mailing paper)

The nucleotide sequence of this 3 cDNA clone was determined by the dideoxynucleotide chain termination method (ABI PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer) and is shown in Figure 2. The DNA fragment has a length of 460 nucleotides. The missing 5' part of the cDNA was isolated using the Marathon TM cDNA Amplification Kit of Clontech (catalog K1802-1) and following the procedure as described in the manual. Briefly, Poly A+ RNA was isolated from nectaries of *Petunia hybrida* flowers. After double stranded cDNA synthesis, adapters were ligated and a 5' RACE reaction was carried out using the adapter primer AP1 supplied in the kit and a gene-specific primer prat 122. The nucleotide sequence of prat 122 is: 5' -gtgggaaggctatgctacaagc-3' (SEQ ID NO:8) (Figure 2). The PCR product was diluted 10x and 1 µl was used in a second 5' RACE reaction with the nested adapter primer supplied by the kit (AP2) and the nested gene-specific primer prat 119 (Figure 2). The nucleotide sequence of prat 119 is: 5'-ccttctccatggactgcaatgcg-3 (SEQ ID NO:9). After gel electrophoreses a fragment of ±850 bp was obtained that hybridised with clone DD18a. The fragment, now called RC8, was extracted from the gel, purified and cloned into a PMOSBlue T-vector as described above. The sequence is shown in Figure 3. The combined (overlapping) sequences of clones DD18a and RC8 are shown in Figure 4, comprising the full length cDNA of a gene called *NEC1* hereafter. The *NEC1* clone has a length of 1205 nucleotides and encodes for a polypeptide of 265 amino acid residues. Based on the deduced amino acid sequence, high homology was found with a cDNA that is associated with *Rhizobium*-induced nodule development in the legume *Medicago trunculata* (MtN3, gene bank number: gn1/PID/e274341). The percentages of identity and similarity are 47% and 72% respectively. Analysis of the predicted protein, using the CAOS/CAMM programme (Protein analysis 1991, Genetics Computer Group inc., Wisconsin USA), shows that the putative protein structure resembles membrane proteins, having six evenly spaced hydrophobic loops that traverse the cell

membrane. In addition, a signal sequence is predicted at the N-terminus, while the C-terminus is highly hydrophilic. Highest homology with MtN3 is found in the N-terminal signal sequence, the first two membrane-spanning loops and the last two membrane-spanning loops. The C-terminal hydrophilic part shows the lowest homology (28% identity, 30% similarity). The function of *NEC1* has not yet been determined.

Please amend the paragraph beginning on page 22, line 17 as follows:

The RNA expression of *NEC1* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete sequence of the Differential Display clone DD18 (Figure 2) was used as a probe. Using 10 μg of total RNA from various petunia tissues, strong expression of *NEC1* was detectable in nectaries and weak expression in anthers. No expression was detectable in other floral organs, in leaves or in roots (Figure 5A).
The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the nucleotides 79 to 1036 of *NEC1* cDNA, comprising the coding region and part of the 3 untranslated region. A clone containing this sequence was obtained by PCR on adapter-ligated cDNA, using two gene-specific primers prat 122 and prat 129 (Figure 4). The nucleotide sequence of prat 122 is: 5' -gtgggaaggctatgctacaagc-3'" (SEQ ID NO:8), comprising the nucleotides 1015 to 1036 of the *NEC1* cDNA. The nucleotide sequence of prat 129 is: 5' - gggatccatggcgcaattacgtgctgatg-3' (SEQ ID NO:10), comprising the nucleotides 79 to 100 of the *NEC1* cDNA. The gene-specific region of the primers is underlined. The primer contains an extra BamHI and NcoI site at the 5' end. A PCR fragment of 958 nucleotides was obtained and cloned into a PMOSBlue vector. The fragment was subcloned in a vector containing the T7 promoter and in vitro antisense RNA transcripts were made using T7

RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. Strong hybridizing signals were observed in the outer cell layers of the nectaries (Figure 6A)

Please amend the paragraph beginning on page 23, line 16 as follows:

The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboraties. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. Genome Walker adapters were ligated and PCR reactions were carried out on each Genome Walker "library" with a gene specific, reversed primer prat 148 and the adapter primer from the kit (AP1). The nucleotide sequence of prat 148 is: 5' - ccaagaaggccaaatatgaaagac-3' (SEQ ID NO:11) comprising the nucleotides 105 to 128 of the *NEC1* cDNA (Figure 4). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer prat 149. The nucleotide sequence of prat 149 is: 5' -aagtcatcagcacgtaattgcgcc-3' (SEQ ID NO:12), comprising the nucleotides 81 to 104 of the *NEC1* cDNA. From the second PCR a 2 kb fragment was isolated from the *Stu*I library, which was cloned in the PMOSBlue T-vector, yielding the construct pMA5-10. Figure 7 (SEQ ID NO:7) shows the DNA sequence of the *NEC1* promoter in the construct pMA5-10, including the translation start of *NEC1* cDNA.

Please amend the paragraph beginning on page 24, line 4 as follows:

A PCR reaction was performed on pMA5-10 (example 5), using the forward vector primer U19 of pMOSBlue and the gene-specific primer prat 169. The nucleotide sequence of prat

169 is:

5' -cgctgcagcgccatggttttttttagtgaagcccc-3' (SEQ ID NO:13). The gene-specific region is underlined. The primer contains an NcoI and BglII restriction site at the 3 end. The PCR product was digested with KpnI and NcoI and ligated into a pBluescript-derived vector (pMO4) that contains the NTM19 promoter (Custers et al., 1997), the reporter gene *GUS* and the nos terminator. The KpnI/NcoI NTM19 promoter fragment was replaced, resulting in a *NEC1*-promoter/*GUS* translational fusion. The resulting plasmid pNEP1 was digested with SmaI to release the *NEC1* promoter/*GUS*/nos fragment and this fragment was ligated into a derivative of the binary plasmid pBIN (Bevan, 1984) yielding the binary plasmid pBNEP1 (Figure 8). pBNEP1 was introduced into *Agrobacterium tumefaciens* strain LBA4404 or C58pMP90 by electroporation. Plasmid DNA from the Agrobacterium transformants was isolated and the structure of the binary vector was verified by restriction analysis and PCR.

Please amend the paragraph beginning on page 26, line 22 as follows:

Honey samples were loaded on an SDS PAGE gel and after electrophoreses the gel was blotted on a PVDF membrane. After staining the CVH29 and CVH50 bands were cut out from the blot and N-terminal sequencing was performed on both proteins. The N-terminal sequence of CVH50 is: SVLDFCVADPSLPDGPAGYSCTEPSTVTSQDF (SEQ ID NO:14). The N-terminal sequence of CVH29 is: SVLDFCVADPS-LPDGPAGYSCKEPAKVTVDDFVFHGLGTA (SEQ ID NO:15). A gene bank homology search (BLAST) showed high amino acid sequence homology (63%) with germin-like proteins isolated from Arabidopsis (Figure 12).

Please amend the paragraph beginning on page 27, line 4 as follows:

Because the germin-like protein CVH29 is excreted in heather nectar it was expected that part of the cDNA encodes a signal sequence. Based on the N-terminal amino acid sequence, degenerated primers were designed. The sequence of the forward primer prat 176 is: 5' - gayttytgygtngcngaycc-3' (SEQ ID NO:16) (y= c or t, n= c, t, a or g). The sequence of the reversed primer prat 177 is: ccrtgraanacraartcrtc (SEQ ID NO:17) (r= g or a). A PCR reaction performed on genomic DNA of heather yielded a 99 bp DNA fragment. The fragment was sequenced and two reversed, gene-specific 5 primers were designed to clone the 5 cDNA by Marathon cDNA racing using the kit and protocol of Clontech laboratories (protocol PT1115-1, Clontech Palo Alto USA). The sequence of gene-specific primer prat 207 that was used is: 5' - ggtgactttagagggctccttgc-3' (SEQ ID NO:18), the sequence of gene-specific nested primer prat 206 is:

5' -gctccttgcaggagtagcctgc-3' (SEQ ID NO:19) (Figure 13). RNA was isolated from open flowers of heather and mRNA was prepared using the Pharmacia quickprep micro mRNA kit. After cDNA synthesis and adapter ligation a PCR reaction was performed, using the adapter primer AP1 and the gene-specific primer prat 207. The PCR product was used for a second PCR, using adapter primer AP2 and the nested gene-specific primer prat 206. A single fragment of around 300 nucleotides was obtained and cloned in a PMOSBlue T-vector. Four clones were sequenced. Figure 14 shows that three clones were identical and one clone had two different nucleotides in the untranslated 5 region. A putative signal sequence of 17 amino acids was identified between the ATG start codon and the first codon of the mature protein CVH29 that was identical in all four clones. The nucleotide sequence of the putative signal sequence (SEQ ID NO:6) is:

5' -atgtttcttccaattctcttcaccatttccctcctcttctcctcctcccatgct-3' .

Please amend the paragraph beginning on page 28, line 5 as follows:

To clone the *NEC1* promoter into a PMOSBlue vector a PCR reaction was carried out on pMA5-10 (example 5) using the forward primer prat 247 and the reversed primer prat 248 (Fig. 7). Prat 247 contains an extra Pst1 restriction site. The *Nde*I restriction site of prat 248 coincides with the ATG translation start of *NEC1*. The nucleotide sequence of prat 247 is: 5' - ggctgcaggagtgttctttgatagaatg-3' (SEQ ID NO:20), the nucleotide sequence of prat 248 is: 5' - cgccatatgttttttttatggaagcccc-3' (SEQ ID NO:21). Gene-specific regions are underlined. A 1,8 kb promoter fragment was obtained and cloned into a pMOSBlue vector, yielding the plasmid pNECP.

Please amend the paragraph beginning on page 28, line 17 as follows:

A DNA molecule encoding the signal sequence CVSP as depicted in SEQ ID NO:6 was produced by synthesis and subsequent annealing of two oligo molecules prat 245 and prat 246. The sequence of prat 245 is: 5' tatgttccttccaattcttttcactatttct-cttcttttctcttcttctcatgcttctgttcttgatttc' 3 (SEQ ID NO:22), the sequence of prat 246 is: 5' gatccgaaatcaagaacagaagcatgagaagaagagaaaagaa-gagaaatagtgaaaagaattggaaggaaca'3 (SEQ ID NO:23). The region encoding the signal sequence CVSP is underlined. To ensure correct cleavage of the signal peptide, the linkers were extended with the coding region for the first five amino acids of the mature germin-like protein (Fig. 13). The codon usage of the signal peptide

sequence was optimised for Arabidopsis. By addition of a BamHI restriction site at the 3 end, 2 extra amino acids were linked in frame to the mature protein. The resulting DNA molecule is shown in Figure 15. The fragment was ligated into a Nde1/BamH1 cut PMOSBlue vector, yielding the plasmid pCVSP.

Please amend the paragraph beginning on page 29, line 4 as follows:

A 250 bp long fragment containing the NOS terminator sequence (NOST) was obtained by PCR, using the forward primer prat 251 and the reversed primer prat 252 on DNA of pRAP 33, which is a pUC 19 derived plasmid. Prat 251 adds a SacI and XhoI site, prat 252 adds a SmaI and EcoRI site. The sequence of prat 251 is: 5' -gggagctcgagtcgttcaaacatttggcaataaag-3' (SEQ ID NO:24). The sequence of prat 252 is: 5' -cgaattcccgggatctagtaacatagatgacac-3' (SEQ ID NO:25). The NOST-specific regions are underlined. The PCR product was cloned into pCR-Script™ Amp SK(+) Cloning Kit (Catalog 21188-21190, Stratagene Al Jolla USA), yielding the plasmid pCR-NOST. pCR-NOST was digested with Sac1 and EcoR1 and the resulting fragment was cloned into the pUC 19 (ClonTech), derived plasmid pUCAP yielding the plasmid pCVNOS.

Please amend the paragraph beginning on page 29, line 19 as follows:

The plasmid pGUSN358 was purchased from Clontech (catalog 6030-1) containing the reporter gene GUS in pUC 119, modified to destroy the N-linked glycosylation site within the 1.814 Kb GUS coding sequence. A PCR reaction was carried out with gene-specific primers prat 249 and prat 250, yielding a fragment that contains the GUS gene coding region and a BamH1

restriction site at the 5 end and a Sac1 restriction site at the 3 end. The sequence of prat 249 is: 5' -ccggatccatgttacgtcctgtagaaacc-3' (SEQ ID NO:26). The sequence of prat 250 is: 5' -gggagctcccaccgaggctgtag-3' (SEQ ID NO:27). The GUS specific regions are underlined. Subsequently, the PCR fragment was digested with BamHI and SacI and ligated into the BamHI/SacI cut plasmid pCVNOS, yielding the plasmid pCV2. A schematic representation of pCV2 is given in Figure 17.

Please amend the paragraph beginning on page 34, line 5 as follows:

PCR primers were designed that hybridise with the cDNA of an invertase gene cloned from *Solanum tuberosum*. The 5 primer 5' -AAGGACTTTAGAGAGACCCGACCACTGCTGG-3' (SEQ ID NO:28) and the 3' primer 5' -AAATGTCTTTGATGCATAATATTTCCCATAATC-3' (SEQ ID NO:29) were used for a PCR reaction on genomic DNA of petunia to yield a fragment of around 420 bp. The fragment was sequenced and cloned into a pMOSBlue vector to used as a probe to screen a petunia nectary-specific cDNA library. Hybridizing phage plaques were purified and cDNAs were retrieved by in vivo excision as described in example 2. The expression of the cDNA s was determined by Northern blotting as described in example 3 and the sequence of a nectary-specific invertase was determined as described in example 2. The invertase gene was amplified using a 5' primer that hybridises with sequences just upstream of the ATG translation start site and a 3' primer that hybridises with sequences just downstream of the translation stop site. Extra restriction enzyme recognition sites were generated to allow cloning of the cDNA in sense (overexpression) or antisense direction into the binary vector pCPO31 as described in example 18. The chimerical

gene constructs are transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that exhibit modified sugar composition in nectar.

Please delete the Sequence Listing appearing on page 39, line 1 - page 47, line 1 in entirety and rewrite as shown attached.

IN THE CLAIMS:

Please amend the following claims.

3. (Amended) An isolated DNA sequence according to claim 1, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID N0:7, or a functional fragment thereof having promoter activity.

5. (Amended) An isolated DNA sequence according to claim 4 having.

a) a nucleotide sequence given in SEQ ID N0:4, or

b) a nucleotide sequence that hybridises with the nucleotide sequence of (a) or with a fragment of (a) under the following hybridisation conditions: pre-hybridisation for lh at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min., or

c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

9. (Amended) An isolated DNA sequence according to claim 8, having:

a) a nucleotide sequence given in SEQ ID N0:6 obtained from a plant of Calluna vulgaris, or

b) a nucleotide sequence that hybridises with the nucleotide sequence given in a), under the following hybridisation conditions: pre-hybridisation for lh at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min., or

c) a nucleotide sequence that has at least 95% homology to the nucleotide sequence of a).

10. (Amended) A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) DNA sequence coding for a protein as defined in claim 4 which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription determination and polyadenylation of an RNA molecule.

13. (Amended) A recombinant double-stranded DNA molecule according to claim 11 wherein the promoter is an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein that has at least 60% homology to the amino acid sequence given in SEQ ID NO:1.

14. (Amended) A recombinant double-stranded DNA molecule according to claim 12 wherein the DNA sequence encoding a signal peptide is an isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

15. (Amended) A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claim 12, wherein the recombinant protein is excreted in nectar,

ii regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

16. (Amended) A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claim 11, wherein the recombinant protein interferes with metabolic pathways in the nectaries,

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

17. (Amended) A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claim 11, wherein the recombinant protein interferes with sink strength of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

18. (Amended) A process for producing a transgenic plant exhibiting a modified nectary development, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claim 11, wherein the recombinant protein interferes with the development of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

19. (Amended) A process for producing honey from modified nectar of transgenic plants, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in claim 11, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,
ii) allowing insects to collect nectar from the transgenic plants and to process the nectar into honey.

20. (Amended) A process for producing a recombinant gene product from honey, comprising:

ii) producing a transgenic plant by introducing in a plant cell a recombinant- double-stranded DNA molecule as defined in claim

12, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

2.2. (Amended) Micro organisms containing DNA sequences according to claim 1.

23. (Amended) Micro organisms containing recombinant DNA molecules according to claim 10.

24. (Amended) A plant cell or plant cell culture transformed with one or more DNA sequences according to claim 1.

25. (Amended) A plant cell or plant cell culture transformed with recombinant DNA molecules according to claim 10.

26. (Amended) A plant consisting essentially of the plant cells of claim 24.

27. (Amended) A transgenic plant obtained by the process of claim 15.

REMARKS

The above amendatory action to the specification is taken to comply with the Sequence Listing requirements of 37 CFR 1.821 - 1.825 and, in particular, to assure that each sequence described in the specification has been assigned an appropriate identifier. Applicants submit herewith a computer readable form copy of the Sequence Listing and the required statements that the paper and computer readable form copies of the Sequence Listing are the same and contain no new matter.

The above amendatory action to the claims is taken to avoid claim fees that would otherwise accrue due to the presence of multiply dependent and to eliminate doubly dependent claims.

Respectfully submitted,

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NEW YORK, NY 10023
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c:\docs\13212pre.oct

IN THE SPECIFICATION:

Please amend the paragraph beginning on page 19, line 21 as follows:

The nucleotide sequence of this 3 cDNA clone was determined by the dideoxynucleotide chain termination method (ABI PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer) and is shown in Figure 2. The DNA fragment has a length of 460 nucleotides. The missing 5' part of the cDNA was isolated using the Marathon TM cDNA Amplification Kit of Clontech (catalog K1802-1) and following the procedure as described in the manual. Briefly, Poly A+ RNA was isolated from nectaries of *Petunia hybrida* flowers. After double stranded cDNA synthesis, adapters were ligated and a 5' RACE reaction was carried out using the adapter primer AP1 supplied in the kit and a gene-specific primer prat 122. The nucleotide sequence of prat 122 is: 5' -gtgggaaggctatgctacaagc-3' (SEQ ID NO:8) (Figure 2). The PCR product was diluted 10x and 1 µl was used in a second 5' RACE reaction with the nested adapter primer supplied by the kit (AP2) and the nested gene-specific primer prat 119 (Figure 2). The nucleotide sequence of prat 119 is: 5' -ccttctccatggactgcaatgcg-3' (SEQ ID NO:9). After gel electrophoreses a fragment of ±850 bp was obtained that hybridised with clone DD18a. The fragment, now called RC8, was extracted from the gel, purified and cloned into a PMOSBlue T-vector as described above. The sequence is shown in Figure 3. The combined (overlapping) sequences of clones DD18a and RC8 are shown in Figure 4, comprising the full length cDNA of a gene called *NEC1* hereafter. The *NEC1* clone has a length of 1205 nucleotides and encodes for a polypeptide of 265 amino acid residues. Based on the deduced amino acid

sequence, high homology was found with a cDNA that is associated with *Rhizobium*-induced nodule development in the legume *Medicago trunculata* (MtN3, gene bank number: gnl/PID/e274341). The percentages of identity and similarity are 47% and 72% respectively. Analysis of the predicted protein, using the CAOS/CAMM programme (Protein analysis 1991, Genetics Computer Group inc., Wisconsin USA), shows that the putative protein structure resembles membrane proteins, having six evenly spaced hydrophobic loops that traverse the cell membrane. In addition, a signal sequence is predicted at the N-terminus, while the C-terminus is highly hydrophilic. Highest homology with MtN3 is found in the N-terminal signal sequence, the first two membrane-spanning loops and the last two membrane-spanning loops. The C-terminal hydrophilic part shows the lowest homology (28% identity, 30% similarity). The function of *NEC1* has not yet been determined.

Please amend the paragraph beginning on page 22, line 17 as follows:

The RNA expression of *NEC1* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete sequence of the Differential Display clone DD18 (Figure 2) was used as a probe. Using 10 µg of total RNA from various petunia tissues, strong expression of *NEC1* was detectable in nectaries and weak expression in anthers. No expression was detectable in other floral organs, in leaves or in roots (Figure 5A).
The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the nucleotides 79 to 1036 of *NEC1* cDNA, comprising the coding region and part of the 3 untranslated region. A clone containing this sequence was obtained by PCR on adapter-ligated cDNA, using two gene-specific primers prat 122 and prat 129 (Figure 4). The nucleotide sequence of prat 122 is: 5' -gtgggaaggctatgctacaagc-

3' (SEQ ID NO:8), comprising the nucleotides 1015 to 1036 of the *NEC1* cDNA. The nucleotide sequence of prat 129 is: 5' - gggatccatggcgcaattacgtgctgatg-3' (SEQ ID NO:10), comprising the nucleotides 79 to 100 of the *NEC1* cDNA. The gene-specific region of the primers is underlined. The primer contains an extra BamHI and NcoI site at the 5' end. A PCR fragment of 958 nucleotides was obtained and cloned into a PMOSBlue vector. The fragment was subcloned in a vector containing the T7 promoter and in vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. Strong hybridizing signals were observed in the outer cell layers of the nectaries (Figure 6A)

Please amend the paragraph beginning on page 23, line 16 as follows:

The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboraties. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. Genome Walker adapters were ligated and PCR reactions were carried out on each Genome Walker "library" with a gene specific, reversed primer prat 148 and the adapter primer from the kit (AP1). The nucleotide sequence of prat 148 is: 5' - ccaagaaggccaaatatgaaagac-3' (SEQ ID NO:11) comprising the nucleotides 105 to 128 of the *NEC1* cDNA (Figure 4). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer prat 149. The nucleotide sequence of prat 149 is: 5' -aagtcatcagcacgtaattgcgcc-3' (SEQ ID NO:12), comprising the nucleotides 81 to 104 of the *NEC1* cDNA. From the second PCR a 2 kb fragment was isolated from the *Stu*I library, which was cloned in the PMOSBlue T-vector, yielding the

construct pMA5-10. Figure 7 (SEQ ID NO:7) shows the DNA sequence of the *NEC1* promoter in the construct pMA5-10, including the translation start of *NEC1* cDNA.

Please amend the paragraph beginning on page 24, line 4 as follows:

A PCR reaction was performed on pMA5-10 (Example 5), using the forward vector primer U19 of pMOSBlue and the gene-specific primer prat 169. The nucleotide sequence of prat 169 is:

5' -cgctgcagcgccatggttttttttagtgaagcccc-3' (SEQ ID NO:13). The gene-specific region is underlined. The primer contains an NcoI and BglII restriction site at the 3 end. The PCR product was digested with KpnI and NcoI and ligated into a pBluescript-derived vector (pMO4) that contains the NTM19 promoter (Custers et al., 1997), the reporter gene *GUS* and the nos terminator. The KpnI/NcoI NTM19 promoter fragment was replaced, resulting in a *NEC1*-promoter/*GUS* translational fusion. The resulting plasmid pNEP1 was digested with SmaI to release the *NEC1* promoter/*GUS*/nos fragment and this fragment was ligated into a derivative of the binary plasmid pBIN (Bevan, 1984) yielding the binary plasmid pBNEP1 (Figure 8). pBNEP1 was introduced into *Agrobacterium tumefaciens* strain LBA4404 or C58pMP90 by electroporation. Plasmid DNA from the Agrobacterium transformants was isolated and the structure of the binary vector was verified by restriction analysis and PCR.

Please amend the paragraph beginning on page 26, line 22 as follows:

Honey samples were loaded on an SDS PAGE gel and after electrophoreses the gel was blotted on a PVDF membrane. After staining the CVH29 and CVH50 bands were cut out from

the blot and N-terminal sequencing was performed on both proteins. The N-terminal sequence of CVH50 is: SVLDFCVADPSLPDGPAGYSCTEPSTVTSQDF (SEQ ID NO:14). The N-terminal sequence of CVH29 is: SVLDFCVADPS-LPDGPAGYSCKEPAKVTVDDFVFHGLGTA (SEQ ID NO:15). A gene bank homology search (BLAST) showed high amino acid sequence homology (63%) with germin-like proteins isolated from Arabidopsis (Figure 12).

Please amend the paragraph beginning on page 27, line 4 as follows:

Because the germin-like protein CVH29 is excreted in heather nectar it was expected that part of the cDNA encodes a signal sequence. Based on the N-terminal amino acid sequence, degenerated primers were designed. The sequence of the forward primer prat 176 is: 5' -gayttytgygtngcngaycc-3' (SEQ ID NO:16) (y= c or t, n= c, t, a or g). The sequence of the reversed primer prat 177 is: ccrtgraanacraartcrtc (SEQ ID NO:17) (r= g or a). A PCR reaction performed on genomic DNA of heather yielded a 99 bp DNA fragment. The fragment was sequenced and two reversed, gene-specific 5 primers were designed to clone the 5 cDNA by Marathon cDNA racing using the kit and protocol of Clontech laboratories (protocol PT1115-1, Clontech Palo Alto USA). The sequence of gene-specific primer prat 207 that was used is: 5' -ggtgactttagagggctccttgc-3' (SEQ ID NO:18), the sequence of gene-specific nested primer prat 206 is:

5' -gctccttgcaggagtagcctgc-3' (SEQ ID NO:19) (Figure 13). RNA was isolated from open flowers of heather and mRNA was prepared using the Pharmacia quickprep micro mRNA kit. After cDNA synthesis and adapter ligation a PCR reaction was performed, using the adapter primer

AP1 and the gene-specific primer prat 207. The PCR product was used for a second PCR, using adapter primer AP2 and the nested gene-specific primer prat 206. A single fragment of around 300 nucleotides was obtained and cloned in a PMOSBlue T-vector. Four clones were sequenced. Figure 14 shows that three clones were identical and one clone had two different nucleotides in the untranslated 5 region. A putative signal sequence of 17 amino acids was identified between the ATG start codon and the first codon of the mature protein CVH29 that was identical in all four clones. The nucleotide sequence of the putative signal sequence (SEQ ID NO:6) is:

5' -atgtttcttccaattctcttcaccatttccctcctcttctcctcctcccatgct-3' .

Please amend the paragraph beginning on page 28, line 5 as follows:

To clone the *NEC1* promoter into a PMOSBlue vector a PCR reaction was carried out on pMA5-10 (example 5) using the forward primer prat 247 and the reversed primer prat 248 (Fig. 7). Prat 247 contains an extra Pst1 restriction site. The *Nde*I restriction site of prat 248 coincides with the ATG translation start of *NEC1*. The nucleotide sequence of prat 247 is: 5' -ggctgcaggagtgttctttgatagaatg-3' (SEQ ID NO:20), the nucleotide sequence of prat 248 is: 5' -cgccatatgttttttatggaagcccc-3' (SEQ ID NO:21). Gene-specific regions are underlined. A 1,8 kb promoter fragment was obtained and cloned into a pMOSBlue vector, yielding the plasmid pNECP.

Please amend the paragraph beginning on page 28, line 17 as follows:

A DNA molecule encoding the signal sequence CVSP as depicted in SEQ ID NO:6 was produced by synthesis and subsequent annealing of two oligo molecules prat 245 and prat 246. The sequence of prat 245 is: 5' tatgttccttccaattcttttcactatttct-cttcttttctcttcttctcatgcttctgttcttgatttc' 3 (SEQ ID NO:22), the sequence of prat 246 is: 5' gatccgaaatcaagaacagaagcatgagaagaagagaaaagaa-gagaaatagtgaaaagaattggaaggaaca'3 (SEQ ID NO:23). The region encoding the signal sequence CVSP is underlined. To ensure correct cleavage of the signal peptide, the linkers were extended with the coding region for the first five amino acids of the mature germin-like protein (Fig. 13). The codon usage of the signal peptide sequence was optimised for Arabidopsis. By addition of a BamHI restriction site at the 3 end, 2 extra amino acids were linked in frame to the mature protein. The resulting DNA molecule is shown in Figure 15. The fragment was ligated into a Nde1/BamH1 cut PMOSBlue vector, yielding the plasmid pCVSP.

Please amend the paragraph beginning on page 29, line 4 as follows:

A 250 bp long fragment containing the NOS terminator sequence (NOST) was obtained by PCR, using the forward primer prat 251 and the reversed primer prat 252 on DNA of pRAP 33, which is a pUC 19 derived plasmid. Prat 251 adds a SacI and XhoI site, prat 252 adds a SmaI and EcoRI site. The sequence of prat 251 is: 5' -gggagctcgagtcgttcaaacatttggcaataaag-3' (SEQ ID NO:24). The sequence of prat 252 is: 5' -cgaattcccgggatctagtaacatagatgacac-3' (SEQ ID NO:25). The NOST-specific regions are underlined. The PCR product was cloned into pCR-Script™ Amp SK(+) Cloning Kit (Catalog 21188-21190, Stratagene Al Jolla USA), yielding the plasmid pCR-

NOST. pCR-NOST was digested with Sac1 and EcoR1 and the resulting fragment was cloned into the pUC 19 (ClonTech), derived plasmid pUCAP yielding the plasmid pCVNOS.

Please amend the paragraph beginning on page 29, line 19 as follows:

The plasmid pGUSN358 was purchased from Clontech (catalog 6030-1) containing the reporter gene GUS in pUC 119, modified to destroy the N-linked glycosylation site within the 1.814 Kb GUS coding sequence. A PCR reaction was carried out with gene-specific primers prat 249 and prat 250, yielding a fragment that contains the GUS gene coding region and a BamH1 restriction site at the 5 end and a Sac1 restriction site at the 3 end. The sequence of prat 249 is: 5' -ccggatccatgttacgtcctgtagaaacc-3' (SEQ ID NO:26). The sequence of prat 250 is: 5' -gggagctcccaccgaggctgtag-3' (SEQ ID NO:27). The GUS specific regions are underlined. Subsequently, the PCR fragment was digested with BamHI and SacI and ligated into the BamHI/SacI cut plasmid pCVNOS, yielding the plasmid pCV2. A schematic representation of pCV2 is given in Figure 17.

Please amend the paragraph beginning on page 34, line 5 as follows:

PCR primers were designed that hybridise with the cDNA of an invertase gene cloned from *Solanum tuberosum*. The 5' primer 5' -AAGGACTTTAGAGAGACCCGACCACTGCTGG-3' (SEQ ID NO:28) and the 3' primer 5' -AAATGTCTTTGATGCATAATATTTCCCATAATC-3' (SEQ ID NO:29) were used for a PCR reaction on genomic DNA of petunia to yield a fragment of around 420 bp. The fragment was

sequenced and cloned into a pMOSBlue vector to used as a probe to screen a petunia nectary-specific cDNA library. Hybridizing phage plaques were purified and cDNAs were retrieved by in vivo excision as described in example 2. The expression of the cDNA s was determined by Northern blotting as described in example 3 and the sequence of a nectary-specific invertase was determined as described in example 2. The invertase gene was amplified using a 5' primer that hybridises with sequences just upstream of the ATG translation start site and a 3' primer that hybridises with sequences just downstream of the translation stop site. Extra restriction enzyme recognition sites were generated to allow cloning of the cDNA in sense (overexpression) or antisense direction into the binary vector pCPO31 as described in example 18. The chimerical gene constructs are transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that exhibit modified sugar composition in nectar.

IN THE CLAIMS:

3 (Amended) An isolated DNA sequence according to claim 1 [or 2], obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID N0:7, or a functional fragment thereof having promoter activity.

5. (Amended) An isolated DNA sequence according to claim 4 having:

a) a nucleotide sequence given in SEQ ID N0:4, or

b) a nucleotide sequence that hybridises with the nucleotide sequence of (a) or with a fragment of (a) under the following hybridisation conditions: pre-hybridisation for lh at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min. [as defined in claim 2], or

c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

9. (Amended) An isolated DNA sequence according to claim 8, having:

a) a nucleotide sequence given in SEQ ID N0:6 obtained from a plant of Calluna vulgaris, or

b) a nucleotide sequence that hybridises with the nucleotide sequence given in a), under the following hybridisation conditions: pre-hybridisation for lh at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min. [as defined in claim 2], or

c) a nucleotide sequence that has at least 95% homology to the nucleotide sequence of a).

10. (Amended) A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) DNA sequence coding for a protein as defined in claim 4 [any of claims 4 to 7] which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription determination and polyadenylation of an RNA molecule.

13. (Amended) A recombinant double-stranded DNA molecule according to claim 11 [or 12] wherein the promoter is an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein that has at least 60% homology to the amino acid sequence given in SEQ ID NO:1. [as defined in any of claims 1-3].

14 (Amended) A recombinant double-stranded DNA molecule according to claim 12 [or 13] wherein the DNA sequence encoding a signal peptide is an isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar. [as defined in claim 8 or 9].

15. (Amended) A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claim 12 [any of claims 12 to 14], wherein the recombinant protein is excreted in nectar,

ii regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

16. (Amended) A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in <u>claim</u> [any of claims] 11 [to 14], wherein the recombinant protein interferes with metabolic pathways in the nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

17. (Amended) A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in <u>claim</u> [any of claims] 11 [to 14], wherein the recombinant protein interferes with sink strength of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

18. (Amended) A process for producing a transgenic plant exhibiting a modified nectary development, comprising:

ii) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in <u>claim</u> [claims] 11 [or 14], wherein the recombinant protein interferes with the development of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

19. (Amended) A process for producing honey from modified nectar of transgenic plants, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in claim [any of claims] 11 [to 14], regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,

ii) allowing insects[, preferably bees,] to collect nectar from the transgenic plants and to process the nectar into honey.

20. (Amended) A process for producing a recombinant gene product from honey, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant- double-stranded DNA molecule as defined in claim [any of claims] 12 [to 14], regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects[, preferably bees,] to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

22. (Amended) Micro organisms containing DNA sequences according to claim [one or more of claims] 1 [to 9].

23. (Amended) Micro organisms containing recombinant DNA molecules according to claim [any of claims] 10 [to 14].

24. (Amended) A plant cell or plant cell culture transformed with one or more DNA sequences according to claim [claims] 1 [to 9].

25. (Amended) A plant cell or plant cell culture transformed with recombinant DNA molecules according to claim [any of] 10 [to 14].

26. (Amended) A plant consisting essentially of the plant cells of claim [claims] 24 [or 25].

27. (Amended) A transgenic plant obtained by the process of claim [any of claims] 15 [to 18].

#9

PATENT
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jantina CREEMERS, et al

Serial No.: 09/743,885 Group No.:

Filed: January 16, 2001 Examiner:

For: PROCESS TO COLLECT METABOLITES FROM MODIFIED NECTAR BY INSECTS

Attorney Docket No.: U-013212-4

Assistant Commissioner Patents and Trademarks
Washington, DC 20231

SECOND PRELIMINARY AMENDMENT

Sir:

In response to the Official Communication of March 12, 2002 and further to the Preliminary Amendment dated October 22, 2001, please further amend the application as follows please amend the above application as follows:

IN THE SPECIFICATION:

Please delete the Sequence Listing appearing on page 39, line 1 - page 47, line 1 in entirety and rewrite as shown attached.

CERTIFICATE OF MAILING (37 CFR 1.8a)

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner of Patents and Trademarks, Washington, DC 20231

CLIFFORD J. MASS
Type or print name of person mailing paper)

Date: March 28, 2002

(Signature of person mailing paper)

SEQUENCE LISTING

<110> CREEMERS, Jantina
ANGENENT, Gerrit
KATER, Martin

<120> Process to collect metabolites from modified nectar by insects

<130> U-13212-4

<140> 09/743885
<141> 2001-01-16

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<170> PatentIn Ver. 2.1

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            20                  25                  30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
        35                  40                  45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
    50                  55                  60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
65                  70                  75                  80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
                85                  90                  95

Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
            100                 105                 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
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Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
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Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
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Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
                165                 170                 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
            180                 185                 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
        195                 200                 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
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Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
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Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
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    50                  55                  60
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 65                  70                  75                  80

Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
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Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
            100                 105                 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
        115                 120                 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
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Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145                 150                 155                 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
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Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
            180                 185                 190

Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
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REMARKS

In response to the Official Communication of March 12, 2002, Applicants request the above amendatory action to the specification to comply with the Sequence Listing requirements of 37 CFR 1.821 - 1.825. Applicants resubmit herewith a computer readable form copy of the Sequence Listing and the required statements that the paper and computer readable form copies of the Sequence Listing are the same and contain no new matter.

Respectfully submitted,

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c:\docs\13212secpre.mar

Process to collect metabolites from modified nectar by insects.

Field of the invention

The present invention relates to isolated, purified DNA sequences which can act as promoters in eukaryotic cells. More specifically, the present invention is related to such DNA sequences which act as promoters to express genes in nectaries of plants. The present invention also relates to chimerical gene constructs comprising a structural or a synthetic gene under the control of a promoter that effects expression of said genes in nectaries. This invention also relates to a process for producing metabolites in honey by allowing insects, preferably bees, to collect and process nectar from plants that excrete said metabolites in nectar or other exudates. Further, this invention relates to plant cells, plants or derivatives therefrom, that express the said chimerical gene.

Background of the invention

Nectaries are nectar secreting organs or tissues that can be located inside (floral) or outside (extrafloral) the flower. The main component of nectar is sugar, the variation between nectars of flowers from different species mainly being the concentration and ratio of glucose, fructose and sucrose (Baker and Baker, 1982). In addition, depending on the plant species, varying amounts of polysaccharides, lipids, organic acids, volatiles, minerals, phosphates, alkaloids, amino acids and proteins have been detected

(Baker and Baker, 1982). Being a specialised sink organ, the nectaries are supplied with sucrose by phloem unloading (Davis et al., 1985, Hagitzer and Fahn, 1992).

The mechanisms of sugar accumulation and nectar secretion have been described for several plant species (Fahn et al., 1979). Sugar transport to the nectaries is achieved by active transport mechanisms and/or osmotic and chemical gradients. In the nectaries of many plants sucrose is converted to glucose and fructose, resulting in a hexose dominant nectar. Part of the hexoses are converted to starch, which is hydrolysed prior to anthesis and nectar secretion. Cell to cell transport of nectar in the nectary parenchyma tissue is mainly symplastic, as demonstrated by the presence of many plasmodesmata between these cells (Fahn et al., 1979). Nectar is secreted from secretory cells via the cell membrane (eccrine secretion) or via the Golgi and endoplasmatic reticulum vesicles (granulocrine secretion). Research on the molecular regulation of nectary development and nectary biochemistry has not been reported.

The main function of floral nectar is to reward pollinating insects. Insects collect nectar to meet their short-term energy requirements. Colony-living honeybees process large quantities of nectar into honey, which is stored in honeycombs of the beehive and is used as food supply during the winter period. Within the bee colony different classes of worker bees cooperate in the honey production process. Foraging bees collect pollen and nectar from the flowers and bring it to the hive. On returning to the hive they give most of it up to household bees. Pollen is used as a protein source, especially to feed the brood. Adult nurse and worker bees use little protein, their capacity to digest proteins being very low (Crailsheim et al., 1993). Honey processing takes place by repeated swallowing and bringing up of the nectar from the honey stomach. In the first process 15% of the water content is lost. This semi-

processed nectar is temporarily stored in a honeycomb cell and taken out later for further processing. The final process includes filtering the honey to discard small particles like pollen grains. Sugar metabolising enzymes (invertase, amylase) are added and the honey is concentrated to an average water content of 20%. Most nectars and honeys only contain traces of protein (<0.2%). However, *Calluna vulgaris* (heather) honey can contain up to 1.8% protein, giving it thixotropic properties (Butler, 1962). It is known that bees add enzymes like invertase to nectar during the honey processing. Therefore, the probability that proteases are also added is very low. Protein digestion does not take place in the honey stomach but in the intestine of the honeybee. However, the ability of adult worker bees to digest proteins is very low, their main requirement being energy which they obtain from nectar. Until now, it was not established which proteins are present in heather honey and whether these originate from floral heather nectar or are added to honey by honeybees.

In the present invention it was established that heather honey contains two unique proteins that originate from floral nectar of heather. Based on these results a production system for proteins in nectar and honey was established.

It is an object of the present invention to show that recombinant proteins can be secreted in nectar of transgenic plants, that this nectar is collected by honeybees and that the bees process this nectar into honey that contains the unaltered protein in a concentrated form.

Definitions

Honey: A substance that contains approximately 80% sugar and varying amounts of other components and that is produced by insects, preferably bees, that collect and process

nectar from floral or extrafloral nectaries, from honeydew, other plant exudates or artificial sugar solutions.

MADS box gene: a gene coding for a transcription factor having a region of 56 amino acids which is homologous to a similar region in the Arabidopsis AGAMOUS protein and Antirrhinum DEFICIENS protein. This region is called the 'MADS box'. At least 50% of the amino acids in this region should be identical to the amino acid composition in the MADS boxes of AGAMOUS and/or DEFICIENS.

Nectary: secretory organ or secretory tissue of plants, located in the flowers (floral nectaries) or outside the flower (extrafloral nectaries) that excrete nectar.

Nectar: sugar containing fluid that is secreted by nectaries. Nectar can also contain substances like minerals, amino acids, proteins, organic acids, volatiles, alkaloids etc.

Recombinant protein: the gene product of a recombinant DNA molecule.

Recombinant DNA molecule: A DNA molecule in which sequences which are not naturally contiguous have been placed next to each other by in vitro manipulations.

Promoter: The DNA region, usually upstream to the coding sequence of a gene, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site.

Summary of the invention

The production of recombinant proteins for pharmaceutical purposes is a growing market. Until now, mainly bacterial and yeast systems have been used for bulk production of proteins. Recently animal production systems have also been

developed. With the availability of efficient transformation techniques for plants, procedures to use plants for the production of proteins are now in progress. In plants, the recombinant proteins are targeted to sink organs like tubers and seeds. A serious draw-back of these production methods is that the recombinant protein can only be obtained after extended, and therefore expensive, purification steps.

The present invention provides a method to produce metabolites, preferably recombinant proteins in honey, which is manufactured by insects, preferably honeybees, that collect floral nectar of transgenic plants. Harvesting of honey is very simple and purification of the protein is very straight forward and requires no advanced purification steps. To give an estimation of the protein yield in a crop like rapeseed, we suggest an average protein production of 2% in honey, as has been found in honey of heather. If one hectare of rapeseed yields 100-500 kilo honey in one season, a yield of 2 to 10 kilo protein can be obtained. In addition, the present invention provides a method to collect metabolites from honey that is derived from non-transgenic plants that secrete these metabolites in nectar. An example are secondary metabolites like acetylandromedol, a diterpine compound, that is excreted in nectar of *Rhododendron arboreum* and *Rhododendron barbatum* and of *Piptanthus nepalensis* (Martini et al., 1990).

This invention provides a gene from petunia, *NEC1*, that is highly expressed in the nectaries of petunia and weakly expressed in the stamens. It also provides another gene from petunia, *FBP15*, that encodes a MADS box protein and which is specifically expressed in the nectaries of petunia. Further, it provides the isolated DNA sequences of the promoters of the *NEC1* and the *FBP15* genes. Furthermore, this invention provides an isolated DNA sequence expressed in nectaries encoding a signal peptide that is translation-

ally fused to a germin-like protein (Lane et al., 1993, Dumas et al., 1995), having the function to target the mature germin-like protein to nectar of heather (*Calluna vulgaris*). This invention gives proof that protein-containing sugar solution is collected by honeybees to produce honey that has a higher protein content than the sugar solution itself, the protein having undergone no qualitative alterations. This invention also proofs that a recombinant protein can be produced in nectar of transgenic plants and that this protein is present in honey produced by honeybees that collected this nectar.

Accordingly, this invention provides an isolated DNA sequence which encodes a protein indicated NEC1 and having the amino acid sequence given in SEQ ID NO:1 of the sequence listing hereafter or homologs of NEC1. A homolog of NEC1 is predominantly expressed in nectaries and/or has at least 60% homology with the amino acid sequence given in SEQ ID NO:1. Further this invention provides an isolated DNA sequence which encodes a protein indicated FBP15 and having the amino acid sequence given in SEQ ID NO:2 of the sequence listing hereafter or a homolog of FBP15. A homolog of FBP15 is specifically expressed in nectaries and belonging to the MADS box family. Furthermore, a homolog is also a gene sequence that has at least 80% homology within the MADS box region and a 60% overall homology with the amino acid sequence given in SEQ ID NO:2. Further this invention provides the characterisation and the isolation of a DNA sequence which encodes a signal peptide indicated "CVSP" (Calluna vulgaris signal peptide), wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

The DNA sequences of the invention can also be characterised in that they comprise the *NEC1* gene and the *FBP15* gene having the nucleotide sequences given in SEQ ID NO:4 and

SEQ ID NO:5 respectively, or a functionally homologous gene or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides, said derived nucleotide sequences being obtainable by hybridisation with the nucleotide sequences given in SEQ ID NO:4 and 5 respectively.

Furthermore, the DNA sequences of the invention can also be characterised in that they comprise signal sequence CVSP having the nucleotide sequence given in SEQ ID NO:6, or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides.

Further, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein.

Furthermore, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.

In a further aspect, the invention provides a protein encoded by any of the above defined DNA sequences. Further, the invention provides processes of producing transgenic plants exhibiting excretion of recombinant proteins in nectar, the expression of the chimerical genes and the targeting of the recombinant proteins being under the control of promoter sequences and a signal sequence as described in this invention. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of

the chimerical genes being under the control of promoter regions upstream of other genes that are expressed in nectaries. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of these proteins being under the control of any signal peptide that affects targeting of a protein in nectar.

Also, the invention provides recombinant double stranded DNA molecules comprising expression cassettes to be used in the above process. Further, the invention provides transgenic bacteria, transgenic plants producing recombinant proteins in nectar, and also plant cells, tissue culture, plant parts or progeny plants derived from said transgenic plants. Finally, the invention provides a process to produce recombinant gene products in honey, produced by bees that collect nectar from transgenic plants and process this nectar into honey.

Brief description of the figures:

Figure 1 shows a polyacrylamide gel with PCR products after Differential Display mRNA amplification. PCR reactions were performed with the oligo-dT primer T12MG in combination with 5 different random primers Ap11-AP15 on cDNA samples of pistils without nectaries (two independent samples), nectaries (two independent samples), leaves and a mixture of sepals (s), petals (p) and stamens (a). Bold arrow depicts the cloned fragment DD18.

Figure 2 is the DNA sequence of the Differential Display RT-PCR clone DD18a. The primers prat 122 and prat 119 that were used for 5' RACE PCR reactions are underlined.

Figure 3 is the DNA sequence of clone RC8, obtained by RACE PCR with gene specific primers prat 122 and prat 119 (Fig. 2) in combination with adapter primers. Primer prat 129 (underlined) is used in the next step together with primer prat 122 to amplify the coding region of the *NEC1* cDNA.

Figure 4 is the full length sequence of *NEC1* cDNA. The translation start (ATG) and translation stop (TAA) are depicted bold.

Figure 5 shows the expression of *NEC1* (A) and *FBP15* (B) in wild type petunia plants (line W115) as determined by Northern blot analysis. Blot A contains total RNA, while blot B is enriched for mRNA. The tissues are indicated as: 1= leaf, 2= sepal, 3= petal, 4= stamen, 5= pistil, 6= nectary. For blot A the HindIII/EcoRI fragment of pDD18a was used as a probe. For blot B the full length cDNA of *FBP15* was used as a probe.

Figure 6 Expression of *NEC1* by in situ localisation of *NEC1* transcripts (A) and activity of the *NEC1* promoter in the nectaries (B) and the stamen (C) as shown by GUS expression driven by the *NEC1* promoter. The GUS assay used for the stamens was incubated overnight without modifications to prevent diffusion (example 8). The GUS assay for the nectaries was incubated for 5 hrs, using an assay mixture to prevent diffusion (example 8). For in situ localisation longitudinal sections of flowers of *Petunia hybrida* were hybridised with digoxigenin-labeled antisense *NEC1* RNA

Figure 7 is the DNA sequence from the promoter region upstream of a sequence encoding the NEC1 protein. Underlined is the translation start of *NEC1* cDNA.

Figure 8 depicts a schematic presentation of the T-DNA region between the borders of the binary vector pBNEP1, containing the *NEC1* promoter (Figure 7), the *GUS* reporter

gene and the nos terminator in pBINPLUS. This vector was used to generate transgenic plants to study the expression of the *NEC1* promoter.

Figure 9 shows the SDS-PAGE separation of proteins that are present in commercial honey samples from different flowers. M= marker, lane 1: wattle bark, lane 2: flower mixture, lane 3: heather, lane 4: clover, lane 5: rapeseed.

Figure 10 shows the SDS-PAGE separation of proteins that are present in commercial honey samples of rapeseed (RH2x, RH10x) and heather (HH2x, HH10x) and of nectar samples of rapeseed (RN2x, RN10x) and heather (HN2x, HN10x). M= molecular weight marker. Two (2x) or ten (10x) fold dilutions were used.

Figure 11 shows the SDS-PAGE separation of proteins present in dilutions of the sugar/BSA feeding solution (A) and of honey from bees that had collected the sugar/BSA solution (B). The dilutions of the sugar/BSA and honey/BSA solution was the same for both gels: 1= 15x, 2= 30x, 3= 60x, 4= 75x, 5= 75x, 6= 90x, 7= 105x, 8= 120x, 9= 135x. M= marker

Figure 12 shows the sequence homology of the N-terminal protein sequence of CVH29, a unique protein present in heather honey and nectar, with a germin-like protein GER1 from a gene bank homology search (BLAST).

Figure 13 shows the deduced DNA sequence of the N-terminal protein sequence of CVH29. The degenerated primers prat 176 and prat 177 are underlined (A). The DNA sequence of the PCR product obtained with prat 176 and prat 177 performed on genomic DNA of heather is shown in B. The gene-specific primers prat 207 and prat 206 used to perform 5'RACE PCR reactions on cDNA from heather flowers are underlined.

Figure 14 shows the DNA sequence of four independent clones obtained by 5'RACE PCR with prat 207 and prat 206 on cDNA of heather flowers. The ATG translation start of the putative signal sequence is boxed. The end of the putative signal sequence and the start of the mature protein are indicated by arrows.

Figure 15 is the sequence of the synthetically produced DNA molecule encoding the signal sequence CVSP (boxed) with linkers.

Figure 16 is the schematic representation of the plasmid pCV1. Not all restriction sites are indicated.

Figure 17 is the schematic representation of the plasmid pCV2. Not all restriction sites are indicated.

Figure 18 is the schematic representation of the plasmid pCV3. Not all restriction sites are indicated.

Figure 19 is the DNA sequence of the full length cDNA of *FBP15*. The translation start (ATG) and translation stop (TAA) are boxed. The MAD-box and K-box region are underlined.

Detailed description of the invention

This invention provides processes of producing transgenic plants that produce recombinant proteins in nectaries and nectar that is collected by foraging honeybees. This invention gives evidence that honeybees process protein containing nectar into honey that contains the unaltered protein in a concentrated form. Subsequently, the desired protein can be purified from the honey.

To express recombinant proteins in nectaries of transgenic plants, a translational fusion of an isolated DNA sequence from a promoter region upstream of a sequence encoding a protein that is expressed in nectaries with a sequence encoding the recombinant protein has to be carried out. Preferably, the isolated DNA sequence from a promoter region is upstream of a sequence that is specifically or highly expressed in nectaries.

The invention relates to a DNA sequence isolated from *Petunia hybrida* that encodes a protein indicated NEC1 or a homologous protein or part thereof. A homologous protein has at least 65% homology with the amino acid sequence given in SEQ ID NO:1. The cDNA sequence of the *NEC1* gene is given in Fig. 4 and in SEQ ID NO:4. The deduced amino acid sequence of the *NEC1* gene is given in SEQ ID NO:1. The *NEC1* gene shows strong expression in the nectaries and in a very localised region of the anther filaments of *Petunia hybrida*. The deduced amino acid sequence of *NEC1* predicts a membrane bound protein. The precise function of the gene has not been elucidated yet, but considering the phenotype of transgenic plants that ectopically express *NEC1* in the leaves, a role in sugar metabolism of *NEC1* is apparent.

The present invention also relates to homologous DNA sequences that can be isolated from other organisms, preferably plants, using standard methods and the already known DNA sequence of the *NEC1* gene. More precisely, it is also possible to use DNA sequences which have a high degree of homology to the DNA sequence of the *NEC1* gene, but which are not completely identical, in the process according to the invention. The use of sequences having homologies between 85 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or

recombination. The DNA sequence shown in SEQ ID NO:4 can also be produced by using DNA synthesis techniques.

The invention also relates to a DNA sequence isolated from *Petunia hybrida* that encodes a MADS box protein indicated FBP15 or a homologous protein or part thereof. The cDNA sequence of *FBP15* is given in SEQ ID NO:5. *FBP15* shows exclusively expression in the nectaries of *Petunia hybrida*. The function of *FBP15* is unknown.

The present invention also relates to homologous DNA sequences that can be isolated from other organisms, preferably plants, using standard methods and the already known DNA sequence of *FBP15*. More precisely, it is also possible to use DNA sequences which have a high degree of homology to the DNA sequence of *FBP15*, but which are not completely identical, in the process according to the invention. The use of sequences having homologies between 85 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:5 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or recombination. The DNA sequence shown in SEQ ID NO:5 can also be produced by using current DNA synthesis techniques.

Further, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein that is expressed in nectaries. Furthermore, this invention provides an isolated DNA sequence from the promoter region upstream of an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary specific sequence encodes a protein compri-

sing the amino acid sequence given in SEQ ID NO:2, or a homologous protein that is expressed in nectaries.

More specifically this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:

a) a nucleotide sequence given in SEQ ID NO:4, or

b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

In a further aspect, the invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:

a) a nucleotide sequence given in SEQ ID NO:5, or

b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8 , or a functional fragment thereof having promoter activity.

Further, this invention provides an isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein

that is expressed in nectaries, targeting of the protein to nectar. More specifically, the DNA sequence comprises the nucleotide sequence given in SEQ ID NO:6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6. The use of sequences having homologies between 95 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:6 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or recombination. The DNA sequence shown in SEQ ID NO:6 can also be produced by using DNA synthesis techniques. The signal peptide CVSP was isolated from nectar of *Calluna vulgaris* flowers and from honey processed by honeybees that collected the nectar. The function of CVSP in heather nectaries is to target the germin-like protein to nectar. The DNA sequence CVSP can also be used to target other proteins to nectar in plant species.

A subject of the present invention is the use of DNA sequences for producing recombinant proteins in nectar of plants, wherein the protein is produced in nectaries and targeted to nectar, and wherein expression in nectaries is achieved by using a DNA sequence consisting of the promoter region upstream of a DNA sequence that is expressed in nectaries, and wherein secretion in nectar is achieved by using a DNA sequence that encodes a signal sequence that targets the recombinant protein to nectar. In a further aspect the present invention relates to processes wherein a recombinant protein is expressed in other plant tissues than the nectaries and wherein the biochemical composition of nectar is changed as a consequence of the recombinant gene expression. The present invention also relates to processes wherein a recombinant protein is expressed in nectaries of a transgenic plant, wherein the biochemical composition of nectar or the nectar secretion is changed as

a consequence of this protein expression. In particular, it relates to processes where the recombinant protein is an enzyme that interferes with the sugar metabolism in nectaries.

The production of a recombinant protein in nectaries and nectar is achieved by integrating into the genome of the plants a recombinant double-stranded DNA molecule comprising an expression cassette having the following constituents and expressing it:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

Such DNA molecules are also subject of the invention. The present invention provides an example of such a DNA molecule that contains the described expression cassettes in the form of plasmid pCV3 (Fig. 18), which comprises the promoter region of the *NEC1* gene from petunia, the signal sequence CVSP from heather, the coding region of the reporter gene *GUS* and the NOS terminator. In principle, any promoter that is active in the nectaries of plants can be used as promoter. The promoter is to ensure that the chosen gene is expressed in nectaries. Also, in principle, any signal sequence that targets the expressed protein to nectar can be used as a signal sequence. The signal sequence is to ensure that the protein is excreted in nectar. Furthermore, any sequence that encodes a recombinant protein in nectaries can be used in the present invention. Preferably, the subject of this invention relates to DNA

sequences that encode proteins to be used for pharmaceutical purposes. It is also possible to use the invention to produce proteins for other purposes, e.g. enzymes for biotests or antioxidants for food additives. Furthermore, it is possible to use the invention to produce metabolites in nectar that attract predators of pest insects or that kill or repel pest insects. In another aspect it is possible to use the invention to produce metabolites in nectar that modify the attractiveness of the plant for pollinating insects or improve the health of pollinating insects.

It is also possible to use DNA sequences that encode proteins that modify the nectar composition or the sink strength of nectaries. This means that the recombinant protein interferes with metabolic pathways in the nectaries, resulting in changed levels of compounds that are already present in nectar, or the formation of new compounds in nectar.

In addition, the present invention also relates to expression cassettes that contain the above mentioned DNA sequences, except for a signal sequence. The recombinant protein is then only expressed in the nectaries, but not targeted to the nectar. Consequently, the expression of the recombinant protein in the nectaries can still affect nectar composition.

In a further aspect, the present invention also relates to expression cassettes that contain DNA sequences coding for a protein that is expressed in other tissues than the nectaries. The expression of the recombinant protein affects changes in the biochemical composition of nectar or in nectar secretion.

Finally, the present invention also relates to non-transgenic plants that produce metabolites in nectar that can be harvested and purified from honey that is produced by honeybees that collect this nectar. Examples for these metabolites are alkaloids, terpines, amino acids, proteins, pigments and volatiles.

A preferred embodiment of the process discussed above provides that the expression cassette is transformed to a plant species that produces nectar. Preferably, the recombinant protein is produced in nectar of plants that are visited by honeybees that collect the nectar. Honeybees collect floral as well as extrafloral nectar. The present invention relates to plants that produce recombinant proteins in floral or extrafloral nectar. In addition, the present invention also relates to plants that produce recombinant proteins in other plant organs, said plant organs producing an exudate that is collected by insects, preferably bees, and processed into honey. A particularly preferred embodiment of the present invention are plants that can be grown under controlled conditions. Controlled conditions are greenhouses or field facilities where transgenic plants can be grown according to the safety rules that are required. Preferably, the controlled conditions are such that bee colonies that perform normal foraging behaviour can be maintained in the same compartment during the flowering period. Preferred plants originate from the *Brassicaceae* family, in particular *Brassica napus*.

Examples

Example 1:
Cloning of *NEC1*

The *NEC1* cDNA was isolated using the mRNA Differential Display system (Genhunter Corporation, Brookline USA). The isolation of total RNA from nectaries, sepals, petals, stamens and pistils from open flowers and from young leaves of *Petunia hybrida* was done according to Verwoerd et al. (1989). Two independent RNA isolations were performed on nectaries as well as on pistils. A DNase treatment was carried out on each RNA sample, using the RNA MessageClean™ Kit (Genhunter Corporation Brookline USA, cat. No. M601). A

reverse transcription reaction was carried out on 0.1 μg RNA of each sample, using the oligo-dT primer T12MG from the Genhunter Kit. Following the protocol, PCR reactions were carried out using the arbritary primers AP11-AP15 in combination with primer T12MG from the Kit. The PCR products were loaded on a sequencing gel and after electrophoresis the gel was blotted on 3M paper, dried and exposed to X-ray film (Figure 1). Two adjacent nectary-specific bands were cut out from the blot and the DNA was purified according to the manual. Reamplification of the fragment was carried out using the oligo-dT primer T12MG and the arbritary primer AP15. After electrophoresis, the PCR product was extracted from the agarose gel by freezing the isolated fragment in liquid nitrogen, followed by centrifugation. DNA was precipitated by adding 1/10 volume 1% HAc, 0.1M $MgCl_2$ and 2.5 volume of 96% ethanol to the supernatant. The pellet was dissolved in 10 μl TE buffer. The fragment, now called DD18a, was cloned into a PMOSBlue T-vector (RPN 1719, Amersham Little Chalfont UK) giving the vector pDD18a.

The nucleotide sequence of this 3' cDNA clone was determined by the dideoxynucleotide chain termination method (ABI PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer) and is shown in Figure 2. The DNA fragment has a length of 460 nucleotides. The missing 5' part of the cDNA was isolated using the Marathon TM cDNA Amplification Kit of Clontech (catalog K1802-1) and following the procedure as described in the manual. Briefly, Poly A+ RNA was isolated from nectaries of *Petunia hybrida* flowers. After double stranded cDNA synthesis, adapters were ligated and a 5'RACE reaction was carried out using the adapter primer AP1 supplied in the kit and a gene-specific primer prat 122. The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctacaagc-3' (Figure 2). The PCR product was diluted 10x and 1 μl was used in a second 5' RACE reaction with the nested adapter primer supplied by the kit (AP2) and the nested gene-specific primer prat 119

(Figure 2). The nucleotide sequence of prat 119 is: 5'-ccttctccatggactgcaatgcg-'3 . After gel electrophoreses a fragment of ±850 bp was obtained that hybridised with clone DD18a. The fragment, now called RC8, was extracted from the gel, purified and cloned into a PMOSBlue T-vector as described above. The sequence is shown in Figure 3. The combined (overlapping) sequences of clones DD18a and RC8 are shown in Figure 4, comprising the full length cDNA of a gene called *NEC1* hereafter. The *NEC1* clone has a length of 1205 nucleotides and encodes for a polypeptide of 265 amino acid residues. Based on the deduced amino acid sequence, high homology was found with a cDNA that is associated with *Rhizobium*-induced nodule development in the legume *Medicago trunculata* (MtN3, gene bank number: gn1/PID/e274341). The percentages of identity and similarity are 47% and 72% respectively. Analysis of the predicted protein, using the CAOS/CAMM programme (Protein analysis 1991, Genetics Computer Group inc., Wisconsin USA), shows that the putative protein structure resembles membrane proteins, having six evenly spaced hydrophobic loops that traverse the cell membrane. In addition, a signal sequence is predicted at the N-terminus, while the C-terminus is highly hydrophilic. Highest homology with MtN3 is found in the N-terminal signal sequence, the first two membrane-spanning loops and the last two membrane-spanning loops. The C-terminal hydrophilic part shows the lowest homology (28% identity, 30% similarity). The function of *NEC1* has not yet been determined.

Example 2:

Cloning of *FBP15*

Petunia MADS box cDNA clones were isolated from a cDNA library made from nectaries of *Petunia hybrida* flowers. The cDNA library was constructed using the lambda ZAP cloning vector (Stratagene, La Jolla USA, catalog nr. 200400-

200402). The library was screened under low stringency hybridisation conditions with a mixed probe comprising the MADS box regions of Floral binding protein gene *FBP2*, *FBP6* and *pMADS3* (Angenent et al., 1993, 1994, Tsuchimoto 1993). The hybridizing phage plaques were purified using standard techniques. Using the in vivo excision method, E.coli clones which contain a double-stranded Bluescript SK- plasmid with the cDNA insertion between the EcoRI and XhoI cleavage site of he polylinker were generated. Cross-hybridisation of the purified clones revealed 3 independent clones that did not cross hybridise with previously isolated FBP cDNA's and which were designated *FBP15*, *FBP16* and *FBP17*. The nucleotide sequence of *FBP15* was determined by the dideoxynucleotide-mediated chain termination method and is depicted in SEQ ID NO:5. The *FBP15* cDNA clone has a length of 1157 nucleotides and encodes a peptide of 222 amino acid residues. All characteristics of a MADS box protein are present in FBP15: a N-terminal located MADS box region which shows a high degree of similarity with other MADS box proteins, and a K-box in the middle of the protein with an alpha helical structure. *FBP15* is most similar to the tobacco MADS box protein NAG1, which is an Agamous homolog and expressed in whorl 3 and 4 (Huang et al., 1996, Mizukami et al., 1996).

Example 3:

Expression of *FBP15*

Expression of *FBP15* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete cDNA of *FBP15* was used as a probe. High stringency hybridisation and washing conditions were used. Using 10 μg of total RNA from various petunia tissues, expression of *FBP15* was only detectable in nectaries. Using 10 μg of mRNA from various tissues, prepared by using the kit and protocol of the Quickprep Micro mRNA Purification

Kit (Pharmacia Biotech), expression of *FBP15* was only detectable in nectaries as shown in Figure 5B.

The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the full length cDNA of *FBP15*. In vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. A hybridizing signal was observed evenly strong in all cells of the nectary tissue.

Example 4:

Expression of *NEC1*

The RNA expression of *NEC1* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete sequence of the Differential Display clone DD18 (Figure 2) was used as a probe. Using 10 μg of total RNA from various petunia tissues, strong expression of *NEC1* was detectable in nectaries and weak expression in anthers. No expression was detectable in other floral organs, in leaves or in roots (Figure 5A).

The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the nucleotides 79 to 1036 of *NEC1* cDNA, comprising the coding region and part of the 3' untranslated region. A clone containing this sequence was obtained by PCR on adapter-ligated cDNA, using two gene-specific primers prat 122 and prat 129 (Figure 4). The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctaca-agc-3'", comprising the nucleotides 1015 to 1036 of the *NEC1* cDNA. The nucleotide sequence of prat 129 is: 5'-gggatccatggcgcaattacgtgctgatg-3', comprising the nucleotides 79 to 100 of the *NEC1* cDNA. The gene-specific region of the primers is underlined. The primer contains an extra

BamHI and NcoI site at the 5'end. A PCR fragment of 958 nucleotides was obtained and cloned into a PMOSBlue vector. The fragment was subcloned in a vector containing the T7 promoter and in vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. Strong hybridizing signals were observed in the outer cell layers of the nectaries (Figure 6A)

Example 5:

Isolation of *NEC1* promoter fragment

The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboraties. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. GenomeWalker adapters were ligated and PCR reactions were carried out on each GenomeWalker "library" with a gene specific, reversed primer prat 148 and the adapter primer from the kit (AP1). The nucleotide sequence of prat 148 is: 5'-ccaagaaggccaaatatgaaagac-3' comprising the nucleotides 105 to 128 of the *NEC1* cDNA (Figure 4). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer prat 149. The nucleotide sequence of prat 149 is: 5'-aagtcatcagcacgtaattgcgcc-3', comprising the nucleotides 81 to 104 of the *NEC1* cDNA. From the second PCR a 2 kb fragment was isolated from the *Stu*I library, which was cloned in the PMOSBlue T-vector, yielding the construct pMA5-10. Figure 7 (SEQ ID NO:7) shows the DNA sequence of the *NEC1* promoter in the construct pMA5-10, including the translation start of *NEC1* cDNA.

Example 6:

Construction of *NEC1* promoter-GUS

A PCR reaction was performed on pMA5-10 (example 5), using the forward vector primer U19 of pMOSBlue and the gene-specific primer prat 169. The nucleotide sequence of prat 169 is:
5'-cgctgcagcgccatggttttttttagtgaagcccc-3'. The gene-specific region is underlined. The primer contains an NcoI and BglII restriction site at the 3' end. The PCR product was digested with KpnI and NcoI and ligated into a pBluescript-derived vector (pMO4) that contains the NTM19 promoter (Custers et al., 1997), the reporter gene *GUS* and the nos terminator. The KpnI/NcoI NTM19 promoter fragment was replaced, resulting in a *NEC1*-promoter/*GUS* translational fusion. The resulting plasmid pNEP1 was digested with SmaI to release the *NEC1* promoter/*GUS*/nos fragment and this fragment was ligated into a derivative of the binary plasmid pBIN (Bevan, 1984) yielding the binary plasmid pBNEP1 (Figure 8). pBNEP1 was introduced into *Agrobacterium tumefaciens* strain LBA4404 or C58pMP90 by electroporation. Plasmid DNA from the Agrobacterium transformants was isolated and the structure of the binary vector was verified by restriction analysis and PCR.

Example 7:

Generation of transgenic Petunia plants

Agrobacterium strain LBA4404 transformants were used to transform *Petunia hybrida* using leaf discs as described by Horsch et al. (1985). After shoot and root induction on kanamycin selection media, plants were transferred to soil in the greenhouse.

Example 8:

Histochemical GUS assay

Different plant parts of Kanamycin-resistant plants transformed with the pBNEP1 construct were analysed for the distribution of β-glucuronidase activity (GUS) using the method described by (Jefferson et al., 1987). In transgenic plants with high expression levels diffusion of reaction products to other tissues was observed. To avoid this spreading a modified GUS assay was used. Briefly, tissues were pre-treated with 90% cold acetone at -20°C for 1 h, then rinsed three times 20' with 100 mM phosphate buffer containing 1 mM potassium ferricyanide. After this treatment the standard GUS assay was performed with the modification that ferricyanide was excluded from the reaction mixture.

Example 9:

Results histochemical GUS assay

In very young flowers (<1,4 cm) no blue staining was observed, in flowers of 2-4 cm weak blue staining of the nectaries was observed. In flowers of (4-6 cm) strong blue staining was observed in the nectaries (figure 6B) and in a very restricted region of the upper part of the anther filaments (Figure 6C). GUS expression was highest in the outer cell layers of the nectary parenchyma. In cross sections of the anther filaments GUS expression was observed in all cells except in the xylem of the inner vascular bundle.

Example 10:

Protein analysis of heather honey and nectar

Samples of pure heather honey, together with samples of rapeseed, clover, wattle bark and lavender honey were diluted, dialysed and loaded on a 12% SDS page gel (Laemmli,

1970). All honey samples showed several identical high molecular weight protein bands. Heather honey contained 2 unique protein bands of 29 and 50 kDa (Figure 9). The proteins were named CVH29 and CVH50 (CVH stands for *Calluna vulgaris* honey). To determine the origin of the proteins, nectar and honey samples of rapeseed and heather were prepared and loaded on a 12% SDS page gel. The high molecular weight protein bands of around 70 kDa that are present in all honey samples were not observed in rapeseed or heather nectar (Figure 10). These proteins are added by honeybees during honey processing. Proteins CVH29 and CVH50 are present in heather honey and heather nectar, but not in nectar of rapeseed. Therefore, it was concluded that CVH29 and CVH50 are secreted in nectar of heather and can be recovered from honey derived from this nectar. The protein concentration in the heather honey we tested was around 0.5%.

Example 11:

N-terminal sequence analysis of CVH29 and CVH50

Honey samples were loaded on an SDS PAGE gel and after electrophoreses the gel was blotted on a PVDF membrane. After staining the CVH29 and CVH50 bands were cut out from the blot and N-terminal sequencing was performed on both proteins. The N-terminal sequence of CVH50 is: SVLDFCVADPS-LPDGPAGYSCTEPSTVTSQDF. The N-terminal sequence of CVH29 is: SVLDFCVADPSLPDGPAGYSCKEPAKVTVDDFVFHGLGTA. A gene bank homology search (BLAST) showed high amino acid sequence homology (63%) with germin-like proteins isolated from Arabidopsis (Figure 12).

Example 12:

Identification signal sequence of CVH29

Because the germin-like protein CVH29 is excreted in heather nectar it was expected that part of the cDNA encodes a signal sequence. Based on the N-terminal amino acid sequence, degenerated primers were designed. The sequence of the forward primer prat 176 is: 5'-gayttyt-gygtngcngaycc-3' (y= c or t, n= c, t, a or g). The sequence of the reversed primer prat 177 is: ccrtgraanacraartcrtc (r= g or a). A PCR reaction performed on genomic DNA of heather yielded a 99 bp DNA fragment. The fragment was sequenced and two reversed, gene-specific 5' primers were designed to clone the 5' cDNA by "Marathon cDNA racing" using the kit and protocol of Clontech laboratories (proto-col PT1115-1, Clontech Palo Alto USA). The sequence of gene-specific primer prat 207 that was used is: 5'-ggtgactttagagggctccttgc-3', the sequence of gene-specific nested primer prat 206 is: 5'-gctccttgcaggagtagcctgc-3' (Figure 13). RNA was isolated from open flowers of heather and mRNA was prepared using the Pharmacia quickprep micro mRNA kit. After cDNA synthe-sis and adapter ligation a PCR reaction was performed, using the adapter primer AP1 and the gene-specific primer prat 207. The PCR product was used for a second PCR, using adapter primer AP2 and the nested gene-specific primer prat 206. A single fragment of around 300 nucleotides was obtained and cloned in a PMOSBlue T-vector. Four clones were sequenced. Figure 14 shows that three clones were identical and one clone had two different nucleotides in the untranslated 5' region. A putative signal sequence of 17 amino acids was identified between the ATG start codon and the first codon of the mature protein CVH29 that was identical in all four clones. The nucleotide sequence of the putative signal sequence (SEQ ID NO:6) is: 5'-atgtttcttccaattctcttcaccatttccctcctcttctcctcctcccatgct-3'.

Example 13:

Construction of an expression cassette for excretion of proteins in nectar

To clone the *NEC1* promoter into a PMOSBlue vector a PCR reaction was carried out on pMA5-10 (example 5) using the forward primer prat 247 and the reversed primer prat 248 (Fig. 7). Prat 247 contains an extra Pst1 restriction site. The *Nde*I restriction site of prat 248 coincides with the ATG translation start of *NEC1*. The nucleotide sequence of prat 247 is: 5'-ggctgcaggagtgttctttgatagaatg-3', the nucleotide sequence of prat 248 is: 5'-cgcca-tatgttttttatggaagcccc-3'. Gene-specific regions are underlined. A 1,8 kb promoter fragment was obtained and cloned into a pMOSBlue vector, yielding the plasmid pNECP.

A DNA molecule encoding the signal sequence CVSP as depicted in SEQ ID NO:6 was produced by synthesis and subsequent annealing of two oligo molecules prat 245 and prat 246. The sequence of prat 245 is: 5'tatgttccttccaattcttttcactatttct-cttcttttctcttcttctcatgcttctgttcttgatttc'3, the sequence of prat 246 is: 5'gatccgaaatcaagaacagaagcatgagaagaagagaaaagaa-gagaaatagtgaaaagaattggaaggaaca'3. The region encoding the signal sequence CVSP is underlined. To ensure correct cleavage of the signal peptide, the linkers were extended with the coding region for the first five amino acids of the mature germin-like protein (Fig. 13). The codon usage of the signal peptide sequence was optimised for Arabidopsis. By addition of a BamHI restriction site at the 3' end, 2 extra amino acids were linked in frame to the mature protein. The resulting DNA molecule is shown in Figure 15. The fragment was ligated into a Nde1/BamH1 cut PMOSBlue vector, yielding the plasmid pCVSP.

pNECP was digested with NdeI and PstI to release the *NEC1* promoter fragment which was cloned into the Pst1/Nde1 cut

pCVSP, yielding the plasmid pCV1. A schematic representation of pCV1 is given in Figure 16.

A 250 bp long fragment containing the NOS terminator sequence (NOST) was obtained by PCR, using the forward primer prat 251 and the reversed primer prat 252 on DNA of pRAP 33, which is a pUC 19 derived plasmid. Prat 251 adds a SacI and XhoI site, prat 252 adds a SmaI and EcoRI site. The sequence of prat 251 is: 5'-gggagctcgagtcgttcaaacatttggcaataaag-3'. The sequence of prat 252 is: 5'-cgaattcccgggatctagtaacatagatgacac-3'. The NOST-specific regions are underlined. The PCR product was cloned into pCR-Script™ Amp SK(+) Cloning Kit (Catalog 21188-21190, Stratagene La Jolla USA), yielding the plasmid pCR-NOST. pCR-NOST was digested with Sac1 and EcoR1 and the resulting fragment was cloned into the pUC 19 (ClonTech), derived plasmid pUCAP yielding the plasmid pCVNOS.

The plasmid pGUSN358 was purchased from Clontech (catalog 6030-1) containing the reporter gene GUS in pUC 119, modified to destroy the N-linked glycosylation site within the 1.814 Kb GUS coding sequence. A PCR reaction was carried out with gene-specific primers prat 249 and prat 250, yielding a fragment that contains the GUS gene coding region and a BamH1 restriction site at the 5'end and a Sac1 restriction site at the 3'end. The sequence of prat 249 is: 5'-ccggatccatgttacgtcctgtagaaacc-3'. The sequence of prat 250 is: 5'-gggagctcccaccgaggctgtag-3'. The GUS specific regions are underlined. Subsequently, the PCR fragment was digested with BamHI and SacI and ligated into the BamHI/SacI cut plasmid pCVNOS, yielding the plasmid pCV2. A schematic representation of pCV2 is given in Figure 17.

pCV1 is digested with PstI and BamHI and the resulting fragment is cloned into the PstI/BamHI cut plasmid pCV2, yielding the plasmid pCV3. A schematic representation of pCV3 is given in Figure 18. pCV3 is digested with AscI and

SmaI and the resulting fragment is cloned into a derivative of the binary plasmid pBIN, yielding the binary plasmid pBCV3. pBCV3 was transferred from *Escherichia coli* to the *Agrobacterium tumefaciens* strain LBA4404 and C58pMP90 by electroporation. The transformed Agrobacterium strain was used to transform Arabidopsis and petunia.

Example 14:

Protein production in nectar

Using the *GUS* reporter gene, GUS activity in nectar of transgenic plants was measured according to the method as described by Jefferson et al., (1987). Briefly, the assay was carried out by measuring the amount of methyl umbelliferone (MU) produced by GUS fluorometrically by emission of light of 455 nm. The absolute emission was corrected for artificial quenching using an internal standard of 1nM MU (Angenent et al., 1993).

Example 15:

Feeding experiments with honeybees

In September 1996 a beehive located outside was supplied with a 25% sucrose solution supplemented with 2% BSA (bovine serum albumin). After 3 weeks the bees had consumed 15 litters of the feeding solution and honey was harvested from the hive. Although the flowering season had mostly past, bees still foraged on flowers to collect nectar outside. Therefore, the honey produced during this period is derived from a mixture of the feeding solution and nectar from flowers. An SDS page protein gel was loaded with dialysed honey samples and sugar/BSA solutions. Figure 11 shows that the protein band of BSA was present in all the samples tested and no qualitative changes were observed in the honey samples compared to the sugar/BSA solutions. The BSA concentration in honey was 1.5 times higher than in the feeding samples, demonstrating that protein is concen-

trated in honey. Honeybees that foraged on the sugar/BSA solution did not show any aberrant behaviour and the colony developed normally.

Example 16:

Process of honey production from transgenic plants

Twohundred and fifty transgenic plants that each produce recombinant protein in nectar were grown in a greenhouse of 25 square meters. The facilities were adjusted according to the safety rules according to European law, including safety measures to prevent in- or outflow of insects. A beehive adjusted for small populations, containing around 200 worker honeybees and a queen, was placed in the greenhouse when the plants were flowering. When a queen is present, she will start laying eggs and larvae will come out. The presence of brood stimulates the bees to collect nectar and process it into honey. After 2-3 weeks bees processed the nectar into honey and stored in sealed cells of the honeycomb. Under the described conditions the amount of honey that can be harvested is 250-1000 grams.

Example 17:

Ablation of nectaries

By introducing the highly sensitive Rnase BARNASE in plant cells, under the control of a tissue-specific promoter, cell ablation can be achieved in very specific tissues or organs. Ablation of nectaries can be applied to decrease the attractiveness of plants for pest insects that forage on the nectar that is secreted by nectaries. In addition, plants without nectaries can be obtained that are more resistant to bacterial and fungal infections. An example is given for the ablation of nectary tissue by expressing bacterial BARNASE in nectaries, using the NEC1 promoter.

Plasmid DNA of pNEP1 (example 6) was digested with KpnI and NcoI to release the 1800 bp *NEC1* promoter fragment. The purified promoter fragment was ligated into a pWP90 derived vector, upstream of the BARNASE-BARSTAR bacterial operon construct (Hartley, 1988). The construct contains a 35SCaMV terminator of polyA signal cauliflower mosaic virus terminator sequence downstream of the BARNASE-BARSTAR operon. The resulting plasmid pWP126 was digested with KpnI/ XhoI to release the *NEC1*-promoter/BARNASE-BARSTAR/CamVpolyA fragment and this fragment was ligated into a pBIN-derived vector pBIN Plus. The recombinant vector was transferred via *Agrobacterium tumefaciens* (LBA4404) to petunia variety W115. Transgenic petunia plants were selected with flowers without nectaries or underdeveloped nectaries.

Many promoters are less specific than can be concluded based on promoter/GUS expression is concluded. Because the bacterial BARNASE is highly cytotoxic at very low concentrations it can be preferred to protect other plant tissues by expression of a ribonuclease inhibitor gene under the control of a weak, constitutive promoter (e.g. NOS promoter) or a tissue-specific promoter that is not active in the tissues where cell ablation is to be achieved (Mariani et al., 1992, Beals et al., 1997).

Example 18:

Ectopic nectary development

MADS box genes regulate floral meristem and floral organ identity. Ectopic expression of MADS box genes can change the developmental fate of floral organs or cells. Transgenic petunia plants ectopically expressing FBP11, an ovule-

specific MADS box gene, develop ovule-like structures on sepals and petals (Colombo et al., 1995). *FBP15* is a nectary-specific MADS box gene, involved in the molecular regulation of nectary development. In petunia nectaries develop at the base of the carpel. Ectopic expression of *FBP15* in petunia may result in the development of nectaries on other organs of the flower or on vegetative parts of the plant. An example is given of a gene construct that, when transformed to a plant, results in ectopic expression of *FBP15*.

FBP15 was amplified using a 5' primer that hybridises with *FBP15* sequences just upstream of the ATG translation start site and a 3' primer that hybridises with *FBP15* sequences just downstream the translation stop site. The 5'primer contains a NcoI recognition site, the 3'primer contains a BamHI recognition site. After the sequence was confirmed, the amplified *FBP15* fragment was inserted as a BamHI/NcoI fragment into the binary vector pCPO31. This binary vector was derived from pPCV708, as described by Florack et al. (1994), and contains three expression cassettes with a multiple cloning site between the left and right T-DNA borders. The cDNA was cloned in sense orientation between a modified CaMV 35S promoter and the nopaline synthase terminator sequence. The chimerical gene construct was transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that show ectopic nectary development.

Example 19:

Modification of sugar composition and nectar secretion

Although sugar content of nectar from different petunia W115 flowers shows some variation, the ratio between hexoses and sucrose is very stable. Down-regulation or up-regulation of genes involved in the establishment of the

ratio between hexoses and sucrose in nectar will therefore modify nectar composition. An example is given for anti-sense expression of a petunia-derived invertase gene.

PCR primers were designed that hybridise with the cDNA of an invertase gene cloned from *Solanum tuberosum*. The 5' primer 5'-AAGGACTTTAGAGAGACCCGACCACTGCTGG-3'and the 3' primer 5'-AAATGTCTTTGATGCATAATATTTCCCATAATC-3' were used for a PCR reaction on genomic DNA of petunia to yield a fragment of around 420 bp. The fragment was sequenced and cloned into a pMOSBlue vector to used as a probe to screen a petunia nectary-specific cDNA library. Hybridizing phage plaques were purified and cDNAs were retrieved by in vivo excision as described in example 2. The expression of the cDNA's was determined by Northern blotting as described in example 3 and the sequence of a nectary-specific invertase was determined as described in example 2. The invertase gene was amplified using a 5' primer that hybridises with sequences just upstream of the ATG translation start site and a 3' primer that hybridises with sequences just downstream of the translation stop site. Extra restriction enzyme recognition sites were generated to allow cloning of the cDNA in sense (overexpression) or antisense direction into the binary vector pCPO31 as described in example 18. The chimerical gene constructs are transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that exhibit modified sugar composition in nectar.

Example 20

Modification of plant development

A DNA which is the *NEC1* gene or a homologous gene is introduced into a plant cell, the said DNA being induced by promoter elements controlling the expression of the introduced DNA in such a way that transcription produces

sense RNA. Plants were regenerated from the transgenic cells as described in example 7. Plants that ectopically express the *NEC1* gene exhibited modified leaf morphology and modified sugar composition. Furthermore, plants that ectopically express the *NEC1* gene showed a delay in flowering time.

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Sequences:

SEQ ID NO:1 amino acid sequence NEC1

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1   MAQLRADDLS FIFGLLGNIV SFMVFLAPVP TFYKIYKRKS SEGYQAIPYM
5
51  VALFSAGLLL YYAYLRKNAY LIVSINGFGC AIELTYISLF LFYAPRKSKI

101 FTGWLMLLEL GALGMVMPIT YLLAEGSHRV MIVGWICAAI NVAVFAAPLS

10  151 IMRQVIKTKS VEFMPFTLSL FLTLCATMWF FYGFFKKDFY IAFPNILGFL

201 FGIVQMLLYF VYKDSKRIDD EKSDPVREAT KSKEGVEIII NIEDDNSDNA

251 LQSMEKDFSR LRTSK
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SEQ ID No: 2 amino acid sequence of FBP15

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Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn
 1               5                  10                  15

Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
            20                  25                  30

Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
        35                  40                  45

Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
    50                  55                  60

Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
65                  70                  75                  80

Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
                85                  90                  95

Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
            100                 105                 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
        115                 120                 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
    130                 135                 140

Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145                 150                 155                 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
                165                 170                 175

Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
            180                 185                 190

Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
        195                 200                 205

Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
    210                 215                 220
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SEQ ID NO:3 amino acid sequence CVSP

MFLPILFTISLLFSSSHA

SEQ ID NO:4 Nucleotide sequence *NEC1*

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1     TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC
51    TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG
101   ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151   TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201   AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251   TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC
301   ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT
351   CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401   TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA
451   GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA
501   TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA
551   CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC
601   TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651   TGCGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT
701   TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT
751   CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA
801   CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT
851   TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC
901   AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA
951   GCTAATTAGC AAGACTTTAG CAGCTTGTAA TATTTAGTGT TTGTGAGGTG
1001  TTACCTTATA ATTAGCTTGT AGCATAGCCT TCCCACTAAT AATTCTGCTT
1051  AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT
1101  ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC
1151  CTCAATTTGT CACTTACTTA TAAGTAGCTG AATAATATAA TGCAATTTTC
1201  ACCCC
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SEQ ID NO:5 Nucleotide sequence *FBP15*

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1     TCTGAATACAAGCTGTGTGTGTAGAGAGATTTCATAAAGACAGCAAACAT
51     CCCTTCTTTTTGTTCTGTTTTAAAAGTTCCCTTCTTCAACCAGCTCTTTT
101   CCTCATCAGGGTAAGTTGCAAATAAAGGGGATGTTCCAGAATCAAGAAGA
151   GAAGATGTCAGACTCGCCTCAGAGGAAGATGGGAAGAGGAAAGATTGAGA
201   TTAAGAGGATTGAAAATACAACAAATCGTCAAGTCACTTTCTGTAAGAGA
251   AGAAATGGGTTGCTTAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGC
301   TGAAGTTGCTCTCATCGTTTTCTCAAGCCGTGGCCGCCTCTATGAATATG
351   CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA
401   GATTCCTCCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA
451   ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA
501   ACAGGAACATGCTAGGCGAGTCTCTAAGTTCTCTGACTGCAAAAGATCTG
551   AAAGGCCTGGAGACCAAACTTGAGAAAGGAATTAGTAGAATTAGGTCCAA
601   AAAGAATGAACTCCTGTTTGCTGAGATTGAGTATATGCGAAAAAGGGAAA
651   TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT
701   GAAAGAAATGTGAACATGATGGGAGGAGAATTTGAGCTGATGCAATCTCA
751   TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACAGCATAATC
801   ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTATAAGTTTAT
851   AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTTGGATTATG
901   TATAAGGGAGTGCAGGCCATTTGCCAATTATTGAAAGGTACTCAAACAGG
951   AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC
1001  TTAGCTTATGGACTCTAAAACAAAGACTTAATTTAACATATAAATATAAT
1051  TGTGTAATGCTGTTGTATTGTATGGTATGTATCCAAAAACATTAATAACC
1101  TATCTTTTTCTTCAAATTATGTCTCCTTTGATACAAACTACTAACATATT
1151  TTCTTAT
```

SEQ ID NO:6 Nucleotide sequence CVSP

ATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCTTCTCCTCCTCCCATGCT

SEQ ID NO:7 Nucleotide sequence *NEC1* promoter

```
1     CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA
51    TGGTAAGGTGAAGGATCTGTTGATTAGAGTTGGGAAGTTCATGTTCTCTG
101   CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT
151   TTGGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAACT
201   TGGGGAGATGACTGTGAGAGCGCATGGAGAAAAGGTTACTTTCAAGGTTT
251   ATAATAAAAAGGATCATATGGCTAAGTTTGAAGAGTGTTCTTTGATAGAA
301   TGTGTCAGACGAGAACATGAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA
351   TGTAGAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT
401   CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTCGTAACAAGAGGAGACGT
451   AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTTAACTAA
501   GGCGCTTGTCGGGAGGCAACCCTAGCTTTGTATGTAAATGTAAAAGTAAA
551   AAATATATATATAGAAAAAGGAAAATACAAAAAGAGTCGTGCCGCGACGT
601   TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT
651   TTTACTTATTTAGTATTACGTAGTTTCTTGTTGTTTTTGTAGGGCTCGGG
701   ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTGTATTGCAGCG
751   AGGTAAGTGTAAGAGTTGAGTTGGAAGCGTTTGGCCAAGTGTTGCACCGT
801   GAGAGGCTTTCAACCTGTTGCGACACGTGAAAAATTAAGAGCCAGATCTG
851   CTACATTAGCACTGAAGCATCGCTTGGCCAATAGCTTGGAATGGAAGCAA
901   GAATTCAAACCAAAATCAGAAACGCCACAAGAGATGTGTCGCACACTGCA
951   AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG
1001  CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCAGCAAAACAGAAAC
1051  GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACAGAACGT
1101  CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG
1151  ACTAGAAAAGCTCTAGCAGATGCGTCGCGTATTGTATAGCTTGGTGTGAA
1201  ACAGAAAGTTCGAAACTTGGAAAACGATAACCCAGCGTCGCCTCTTCAAC
1251  CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG
1301  ATCGGCTGATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT
1351  CATAAGTTTGACCTAGGATATTTTATATATATATATATATATATACACAC
1401  ACACACCATTTCCAGCGATCTTACCTCATTTTTATTCAAACCATTTTTCT
```

1451 GCTTCAAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA

1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA

1551 TGGTAAGATAAACATTGTTTGCAAGTGTACAATTTTAGTATATGCAAACC

1601 AACGCTTCTTCTTCCAACTATCACCTAAAACTACATCATTTATGGCGGGC

1651 GGACTAGACGTAGCCAAATATAAAAACGCAATGGCCATTCAGTTCATGTC

1701 ATTTTTATATCCTTCATCCAATAATATTACTCAAAATTGATGTACAGTTT

1751 GGTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT

1801 TAAAGAGAACTGTTCCACTAAATTTTAATGATTTAATTAATTTAACTCGG

1851 TTACTTGTATTATTATTATTGCTGTATTTGTTTGTCATTTGAATTTGGCA

1901 CCGCAGATTTTTGTATGCAATTAACCCTCATATATCTTTTGGCCAAATAA

1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT

2001 TCTTGTTTTTTTGTTTCTCTGTTGTTGTTCTCCACTATAAATAACATTTGC

2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTTGTAGATTCAACAAGAGTATTC

2101 AGCACTTGAACTCAAAAGGGGCTTCACTAAAAAAAATCATG

SEQ ID NO:8 Nucleotide sequence *FBP15* promoter

CLAIMS

1. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein that has at least 60% homology to the amino acid sequence given in SEQ ID NO:1.

2. An isolated DNA sequence according to claim 1, wherein the nectary-specific expressed sequence has:
   a) a nucleotide sequence given in SEQ ID NO:4, or
   b) a nucleotide sequence which hybridises with (a) or with a fragment of (a) under the following conditions: pre-hybridisation for 1h at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min., or
   c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

3. An isolated DNA sequence according to claim 1 or 2, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

4. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein having at least 60% homology with the amino acid sequence given in SEQ ID NO:1, which protein, when ectopically expressed, plays a role in sugar metabolism, the expression of the DNA sequence being predominantly confined to the nectarics of a plant.

5. An isolated DNA sequence according to claim 4 having:
   a) a nucleotide sequence given in SEQ ID NO:4, or

b) a nucleotide sequence that hybridises with the nucleotide sequence of (a) or with a fragment of (a) under the hybridisation conditions as defined in claim 2, or

c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

6. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination, wherein the DNA sequence encodes a protein exhibiting the same function as the protein according to claim 4.

7. An isolated DNA sequence according to claim 4 having a nucleotide sequence given in SEQ ID NO:4, said sequence being produced by current DNA synthesis techniques.

8. An isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

9. An isolated DNA sequence according to claim 8, having:

a) a nucleotide sequence given in SEQ ID NO:6 obtained from a plant of *Calluna vulgaris*, or

b) a nucleotide sequence that hybridises with the nucleotide sequence given in a), under the hybridisation conditions as defined in claim 2, or

c) a nucleotide sequence that has at least 95% homology to the nucleotide sequence of a).

10. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) a DNA sequence coding for a protein as defined in any of

claims 4 to 7 which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription determination and polyadenylation of an RNA molecule.

11. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence coding for a protein which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

12. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

13. A recombinant double-stranded DNA molecule according to claim 11 or 12 wherein the promoter is as defined in any of claims 1-3.

14. A recombinant double-stranded DNA molecule according to claim 12 or 13 wherein the DNA sequence encoding a signal peptide is as defined in claim 8 or 9.

15. A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 12 to 14, wherein the recombinant protein is excreted in nectar,

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

16. A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:
i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 11 to 14, wherein the recombinant protein interferes with metabolic pathways in the nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

17. A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:
i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 11 to 14, wherein the recombinant protein interferes with sink strength of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

18. A process for producing a transgenic plant exhibiting a modified nectary development, comprising:
i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claims 11 or 14, wherein the recombinant protein interferes with the development of nectaries,
ii) regenerating plants from the transgenic cell, and
iii) selecting transgenic plants.

19. A process for producing honey from modified nectar of transgenic plants, comprising:
i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 11 to 14, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,
ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey.

20. A process for producing a recombinant gene product from honey, comprising:
i) producing a transgenic plant by introducing in a plant cell

a recombinant double-stranded DNA molecule as defined in any of claims 12 to 14, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

21. A process for producing a metabolite from honey, comprising:

i) producing a plant that excretes this metabolite in nectar and which plant has been produced by current breeding and selection methods,

ii) allowing insects, preferably bees, to collect nectar from the selected plants and to process the nectar into honey, and

iii) isolating and purifying the metabolite from the honey.

22. Micro organisms containing DNA sequences according to one or more of claims 1 to 9.

23. Micro organisms containing recombinant DNA molecules according to any of claims 10 to 14.

24. A plant cell or plant cell culture transformed with one or more DNA sequences according to claims 1 to 9.

25. A plant cell or plant cell culture transformed with recombinant DNA molecules according to any of 10 to 14.

26. A plant consisting essentially of the plant cells of claims 24 or 25.

27. A transgenic plant obtained by the process of any of claims 15 to 18.

28. Seeds, tissue culture, plant parts or progeny plants derived from a transgenic plant according to claim 27.

29. Honey obtained from nectar from transgenic plants, which nectar has a modified composition.

30. Honey obtained from nectar from transgenic plants, which nectar comprises a recombinant gene product.

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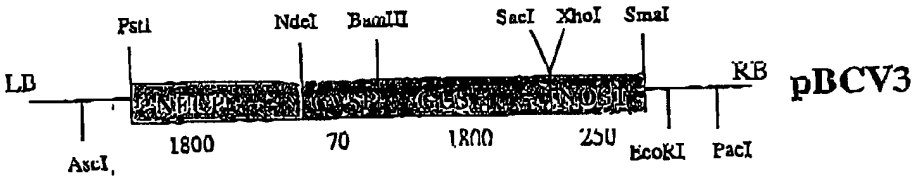
Published

With international search report

(54) Title: PROCESS TO COLLECT METABOLITES FROM MODIFIED NECTAR BY INSECTS



(57) Abstract

The invention relates to a recombinant double stranded DNA molecule comprising an expression cassette comprising the following constituents: i) a promoter functional in nectaries of plants, ii) a DNA sequence encoding a protein which is fused to the promoter, iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule. The invention further relates to a process for producing a recombinant gene product from honey, comprising: i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar, ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and iii) isolating and purifying the gene product from the honey.

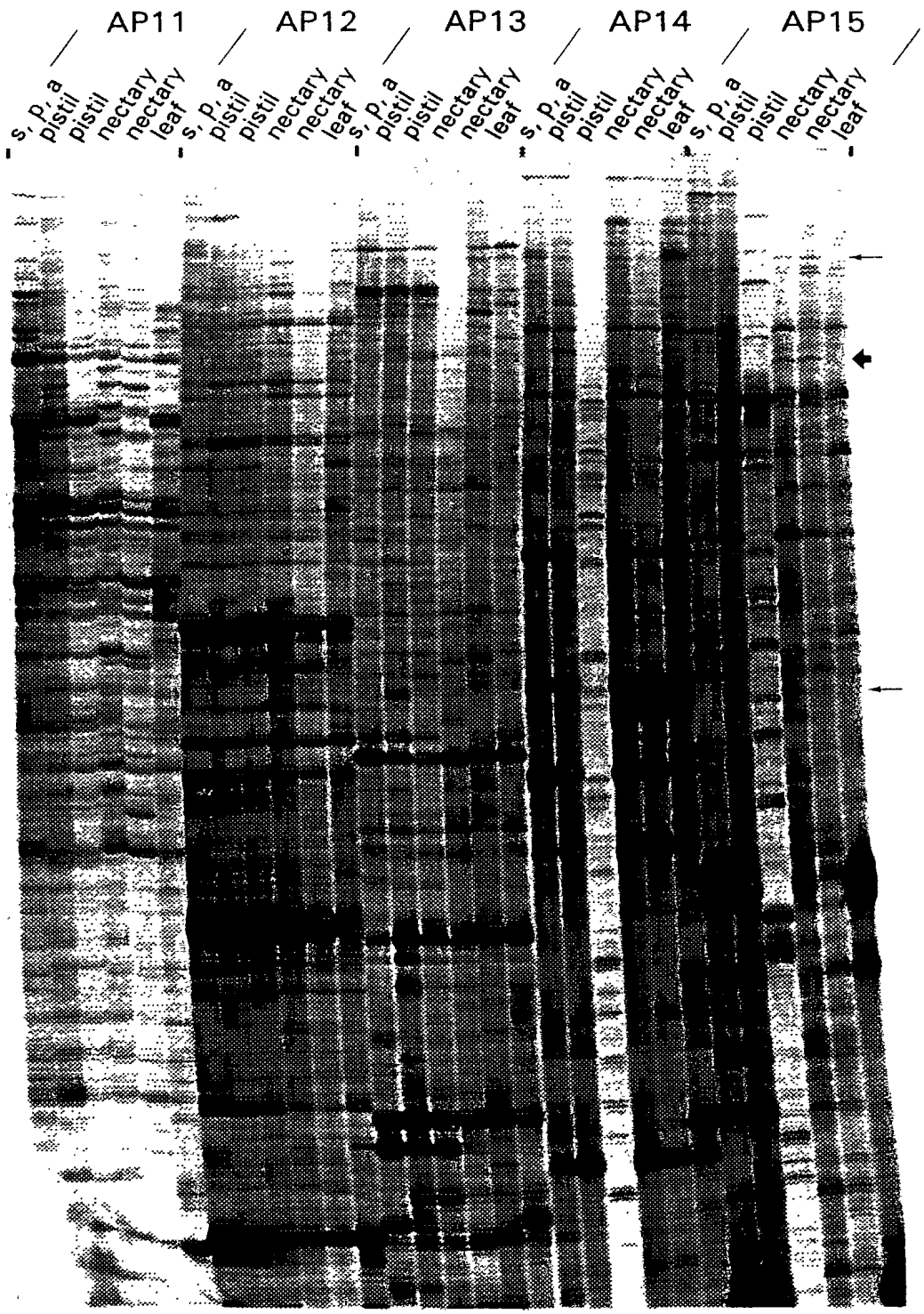

AP11
AP12
AP13
AP14
AP15
s, p, a
pistil
pistil
nectary
nectary
leaf

FIG.1

```
1     TGATCCTGTT CGAGAAGCTA CAAAATCAAA AGAAGGTGTA GAAATCATTA
51    TCAACATTGA AGATGATAAT TCTGATAACG CATTGCAGTC CATGGAGAAG  prat 119 ←
101   GATTTTTCCA GACTGCGGAC ATCAAAATAA GCAAGAAGAT GATCAAAAAA
151   TGACAAAGCT AAGGAGTTTG AAGTAAGGCA AGGAACTTGA CACTGAATAT
201   CTAAGCTAAT TAGCAAGACT TTAGCAGCTT GTAATATTTA GTGTTTGTGA
251   GGTGTTACCT TATAATTAGC TTGTAGCATA GCCTTCCCAC TAATAATTCT  prat 122 ←
301   GCTTAGCGAA TCTTATATAT GGGAAATACT TACACTAGTA TGCATCTTCT
351   ATATACATGT TTGGCACTTG ACTATACATA GAAAAATTAA CAAGCATTTC
401   TCACCTCAAT TTGTCACTTA CTTATAAGTA GCTGAATAAT ATAATGCAAT
451   TTTCACCCC
```

FIG. 2

```
1     TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC
51    TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG prat 129 ⟶
101   ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151   TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201   AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251   TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC
301   ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT
351   CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401   TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA
451   GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA
501   TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA
551   CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC
601   TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651   TGCGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT
701   TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT
751   CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA
801   CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGG
```

FIG.3

```
1     TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC
51    TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG
101   ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151   TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201   AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251   TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC
301   ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT
351   CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401   TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA
451   GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA
501   TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA
551   CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC
601   TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651   TGCGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT
701   TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT
751   CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA
801   CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT
851   TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC
901   AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA
951   GCTAATTAGC AAGACTTTAG CAGCTTGTAA TATTTAGTGT TTGTGAGGTG
1001  TTACCTTATA ATTAGCTTGT AGCATAGCCT TCCCACTAAT AATTCTGCTT
1051  AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT
1101  ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC
1151  CTCAATTTGT CACTTACTTA TAAGTAGCTG AATAATATAA TGCAATTTTC
1201  ACCCC
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FIG. 4

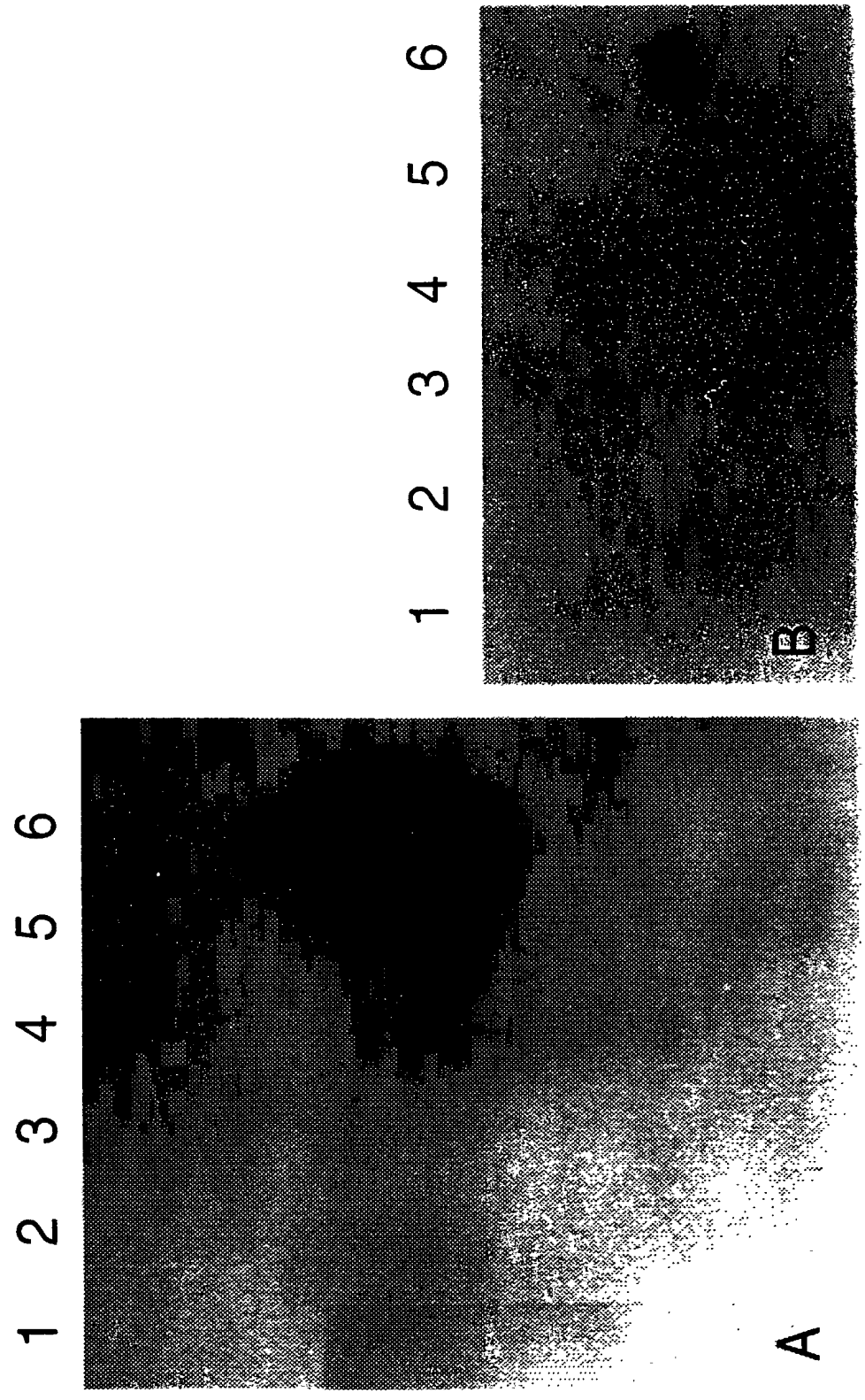

FIG.5

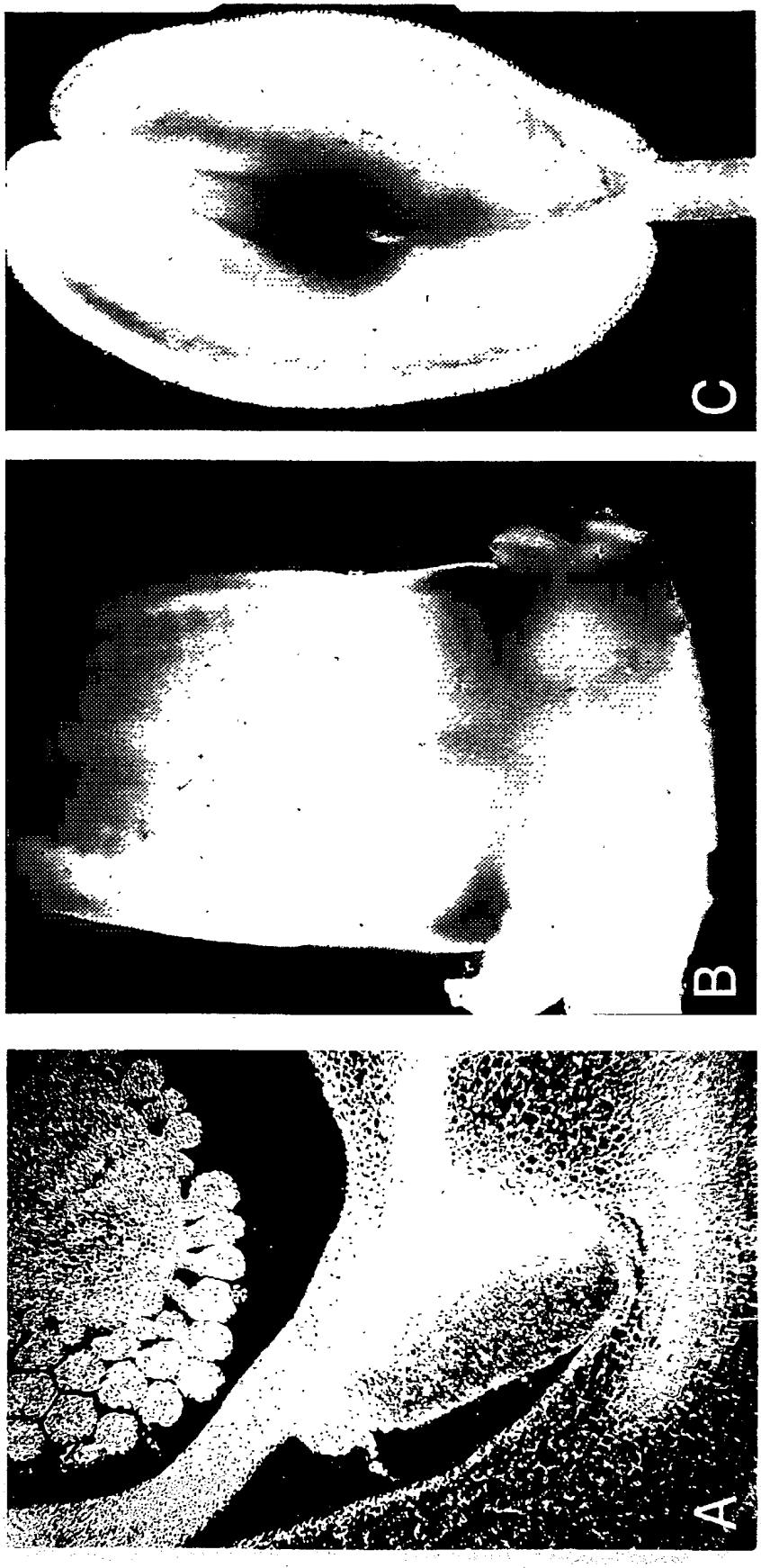

FIG.6

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1       CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA
51      TGGTAAGGTGAAGGATCTGTTGATTAGAGTTGGGAAGTTCATGTTCTCTG
101     CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT
151     TTGGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAACT
201     TGGGGAGATGACTGTGAGAGCGCATGGAGAAAAGGTTACTTTCAAGGTTT
251     ATAATAAAAAGGATCATATGGCTAAGTTTGAAGAGTGTTCTTTGATAGAA
301     TGTGTCAGACGAGAACATGAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA
351     TGTAGAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT
401     CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTCGTAACAAGAGGAGACGT
451     AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTTAACTAA
501     GGCGCTTGTCGGGAGGCAACCCTAGCTTTGTATGTAAATGTAAAAGTAAA
551     AAATATATATATAGAAAAAGGAAAATACAAAAAGAGTCGTGCCGCGACGT
601     TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT
651     TTTACTTATTTAGTATTACGTAGTTTCTTGTTGTTTTTGTAGGGCTCGGG
701     ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTGTATTGCAGCG
751     AGGTAAGTGTAAGAGTTGAGTTGGAAGCGTTTGGCCAAGTGTTGCACCGT
801     GAGAGGCTTTCAACCTGTTGCGACACGTGAAAAATTAAGAGCCAGATCTG
851     CTACATTAGCACTGAAGCATCGCTTGGCCAATAGCTTGGAATGGAAGCAA
901     GAATTCAAACCAAAATCAGAAACGCCACAAGAGATGTGTCGCACACTGCA
951     AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG
1001    CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCAGCAAAACAGAAAC
1051    GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACAGAACGT
1101    CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG
1151    ACTAGAAAAGCTCTAGCAGATGCGTCGCGTATTGTATAGCTTGGTGTGAA
1201    ACAGAAAGTTCGAAACTTGGAAAACGATAACCCAGCGTCGCCTCTTCAAC
1251    CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG
1301    ATCGGCTGATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT
1351    CATAAGTTTGACCTAGGATATTTTATATATATATATATATATATACACAC
```

FIG.7

1401 ACACACCATTTCCAGCGATCTTACCTCATTTTTATTCAAACCATTTTTCT

1451 GCTTCAAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA

1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA

1551 TGGTAAGATAAACATTGTTTGCAAGTGTACAATTTTAGTATATGCAAACC

1601 AACGCTTCTTCTTCCAACTATCACCTAAAACTACATCATTTATGGCGGGC

1651 GGACTAGACGTAGCCAAATATAAAAACGCAATGGCCATTCAGTTCATGTC

1701 ATTTTTATATCCTTCATCCAATAATATTACTCAAAATTGATGTACAGTTT

1751 GGTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT

1801 TAAAGAGAACTGTTCCACTAAATTTTAATGATTTAATTAATTTAACTCGG

1851 TTACTTGTATTATTATTATTGCTGTATTTGTTTGTCATTTGAATTTGGCA

1901 CCGCAGATTTTTGTATGCAATTAACCCTCATATATCTTTTGGCCAAATAA

1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT

2001 TCTTGTTTTTTGTTTCTCTGTTGTTGTTCTCCACTATAAATAACATTTGC

2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTTGTAGATTCAACAAGAGTATTC

2101 AGCACTTGAACTCAAAAGGGGCTTCACTAAAAAAAATCATG

FIG.7(CONTIN.)

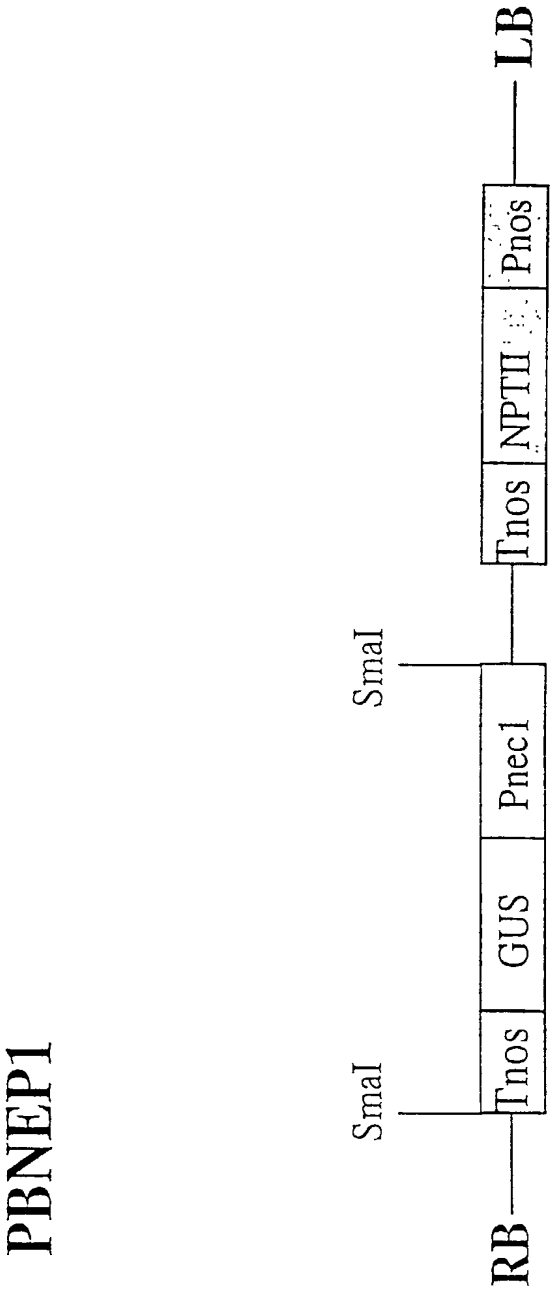

FIG. 8

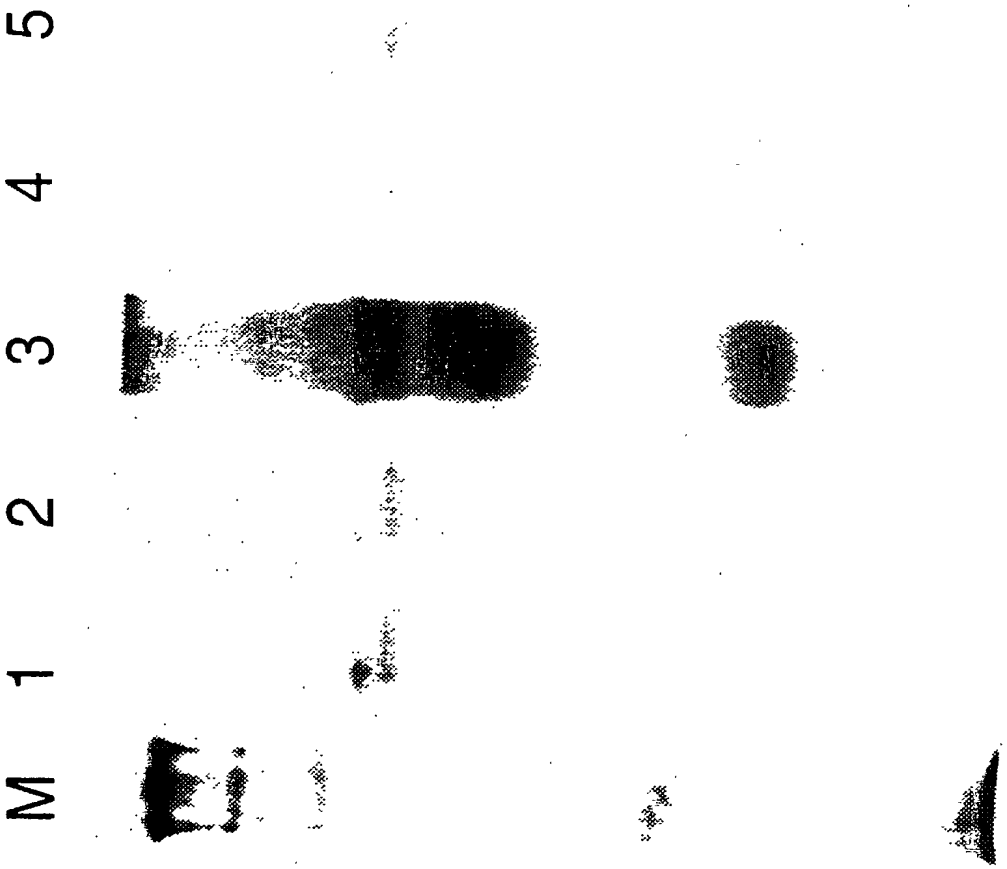

FIG. 9

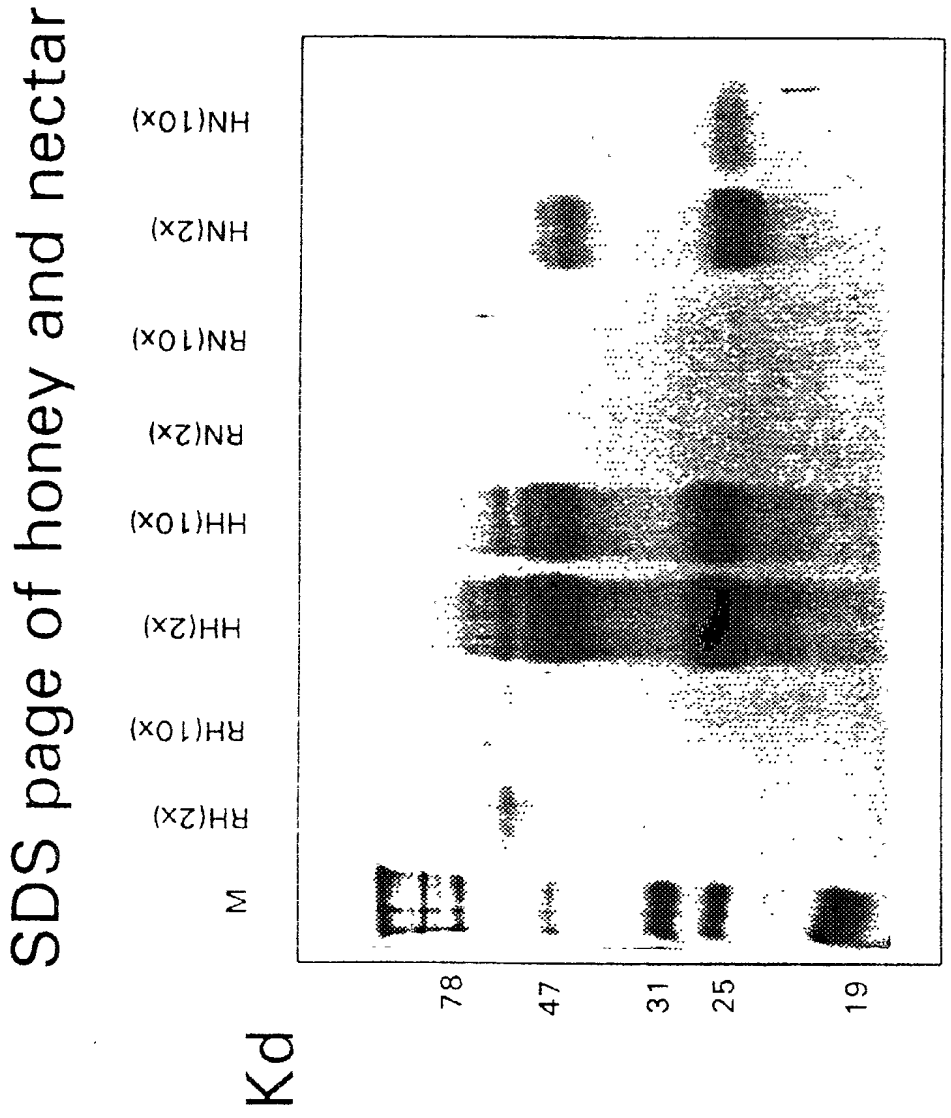
SDS page of honey and nectar
M
RH(2x)
RH(10x)
HH(2x)
HH(10x)
RN(2x)
RN(10x)
HN(2x)
HN(10x)
Kd
78
47
31
25
19

FIG.10

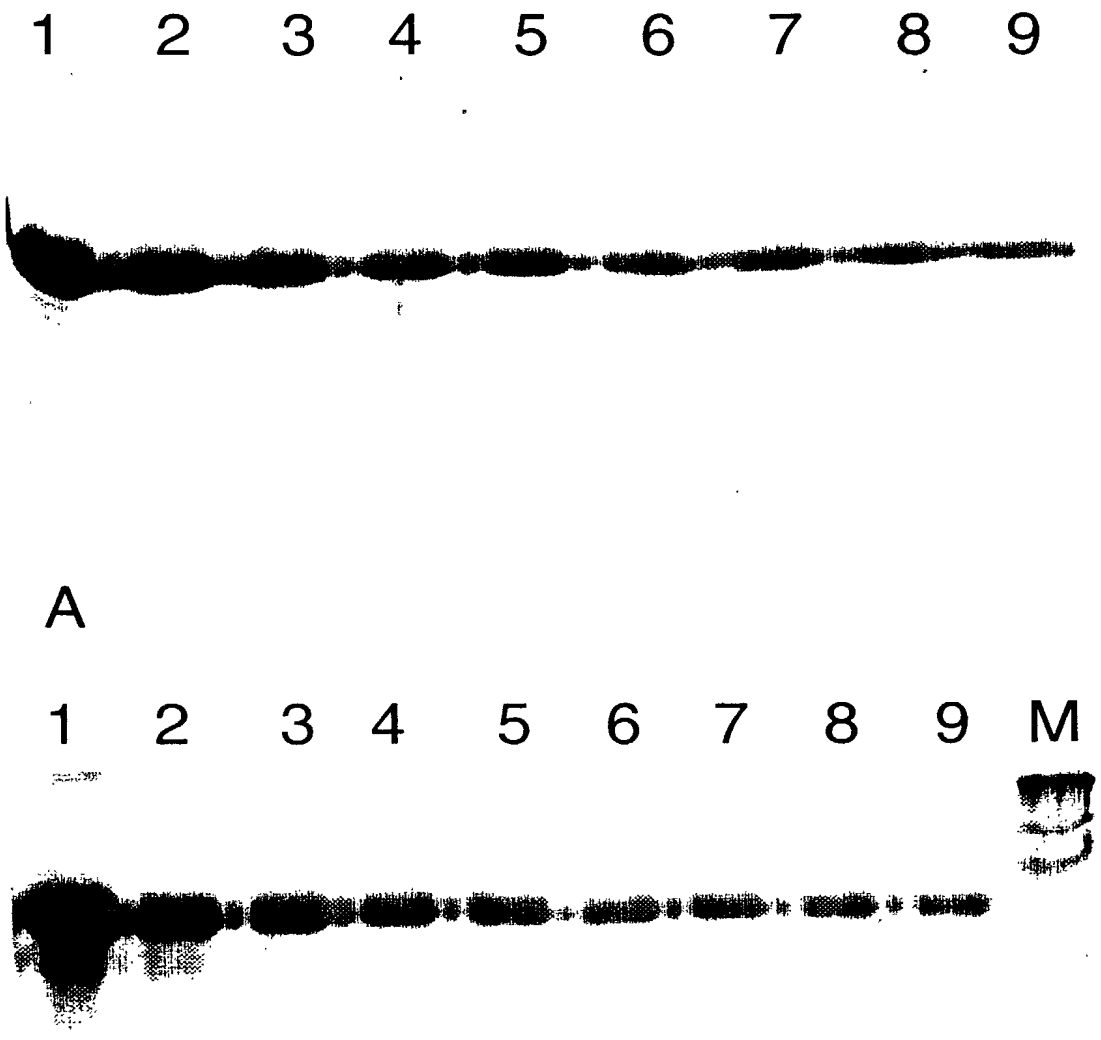

FIG. 11

```
===17-APR-1998============================================================PC/GENE===

**************************************
* ALIGNMENT OF TWO PROTEIN SEQUENCES. *
**************************************

The two sequences to be aligned are:

PCVH29.
Total number of residues: 60.

GER1.
Total number of residues: 211.

Comparison matrix  : Structure-genetic matrix.
Open gap cost      : 7
Unit gap cost      : 1

The character to show that two aligned residues are identical is '|'

PCVH29     - MKMFLPILFTISLLFSSSHASVLDFCVADPSLPDGPAGYSCKEPAKVTVD -50
             |||    | |  ||  | | ||| |||||||  |  | |||||  |  ||
GER1       - MKMRIQIFFILSLFSSISFASVQDFCVADPKGPQNPSGYSCKNPDQVTEN -50

PCVH29     - DFVFHGLGTA                                         -60
             || | ||| |
GER1       - DFAFSGLGKAGNTSNVIKAAVTPAFAPAFAGLNGLDVSLARLDLAGGGVI -100

GER1       - PLHTHPGASEVLVVIQGTICAGFISSANKVYLKTLSRGDSMVFPQGLLHF -150

GER1       - QLNSGKGPALAFVAFGSSSPGLQILPFALFANDLPSELVEATTFLSDEEV -200

GER1       - KKLKGVLGGTN -211


Identity  : 36 ( 60%)
Number of gaps inserted in PCVH29: 0
Number of gaps inserted in GER1: 0

===17-APR-1998============================================================PC/GENE===
```

FIG. 12

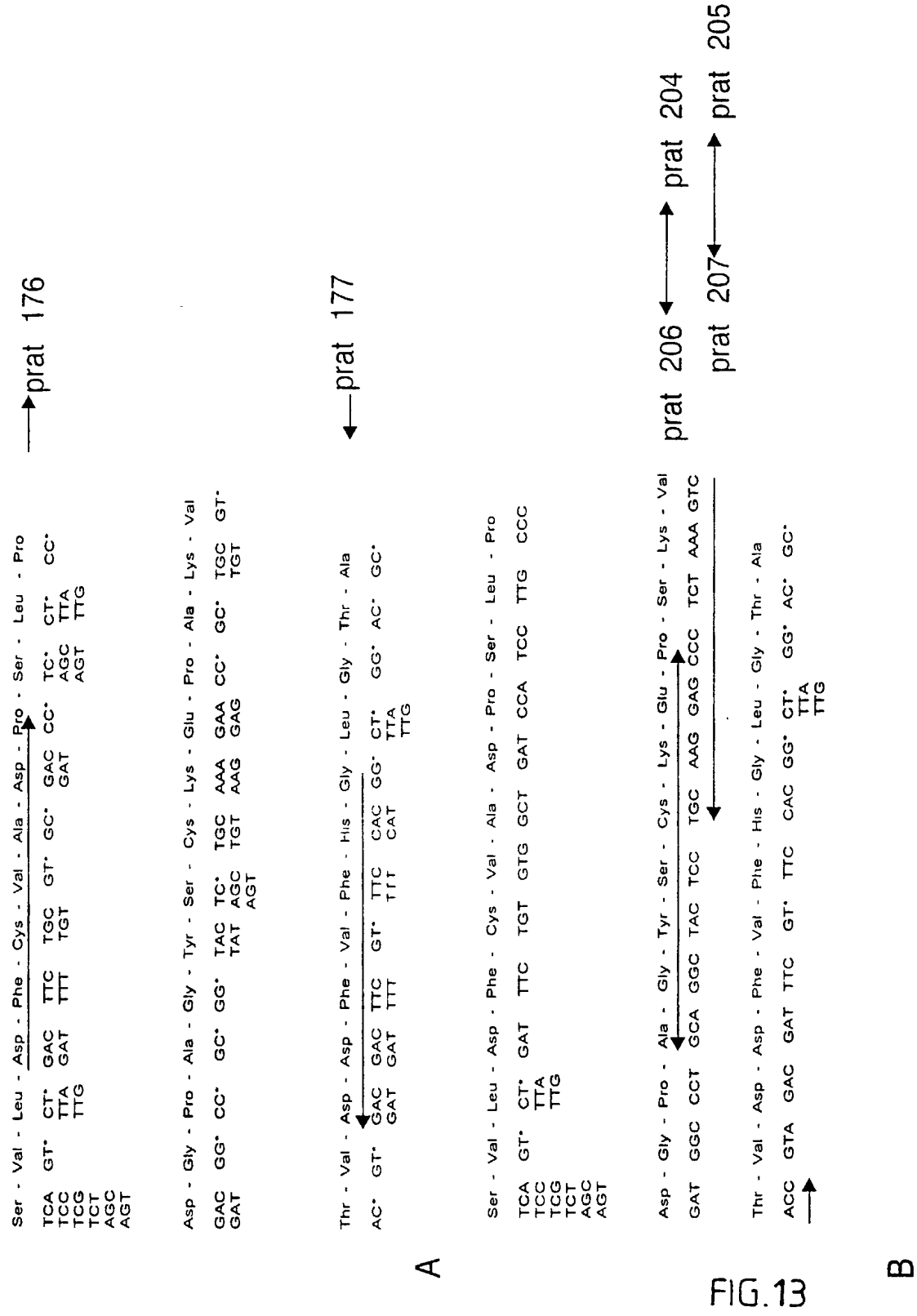

FIG.13

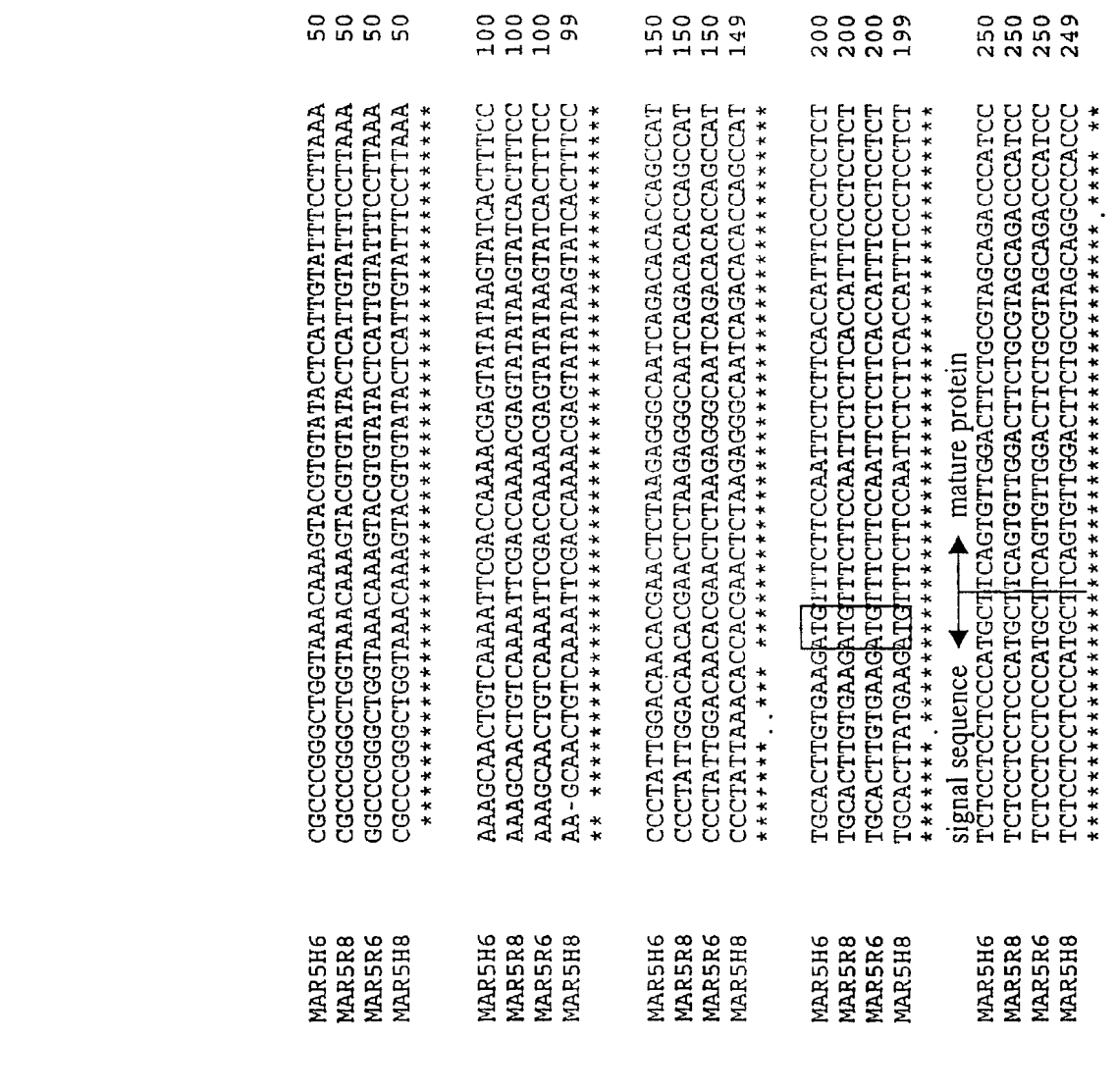
MAR5H6 CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA 50
MAR5R8 CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA 50
MAR5R6 GGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA 50
MAR5H8 CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA 50
MAR5H6 AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC 100
MAR5R8 AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC 100
MAR5R6 AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC 100
MAR5H8 AA-GCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC 99
MAR5H6 CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT 150
MAR5R8 CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT 150
MAR5R6 CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT 150
MAR5H8 CCCTATTAAACACCACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT 149
MAR5H6 TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT 200
MAR5R8 TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT 200
MAR5R6 TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT 200
MAR5H8 TGCACTTATGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT 199
signal sequence
mature protein
MAR5H6 TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC 250
MAR5R8 TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC 250
MAR5R6 TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC 250
MAR5H8 TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGGCCCACCC 249

FIG. 14

tatgttccttccaattcttttcactatttctcttcttttctcttcttctcatgcttctgttcttgatttcg
acaaggaaggttaagaaaaagtgataaagagaagaaaagagaagaagagtacgaagacaagaactaaagcctag

FIG.15

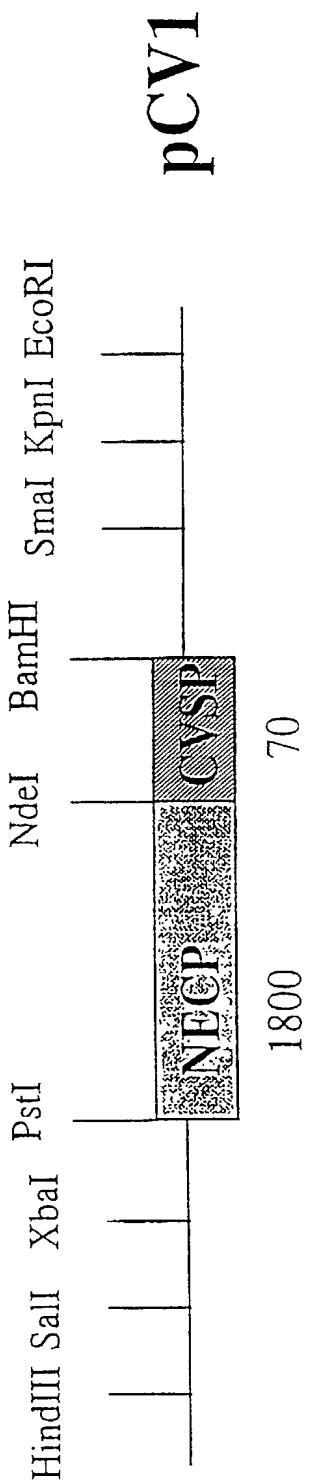
pCV1
HindIII SalI XbaI PstI NdeI BamHI SmaI KpnI EcoRI
NECP
CVSP
1800
70

FIG.16

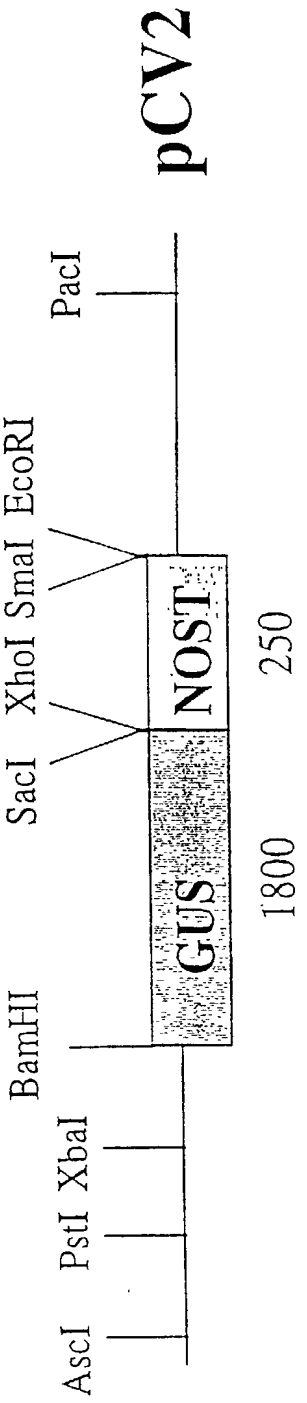
pCV2
PacI
EcoRI
SmaI
XhoI
SacI
NOST
250
GUS
1800
BamHI
XbaI
PstI
AscI

FIG.17

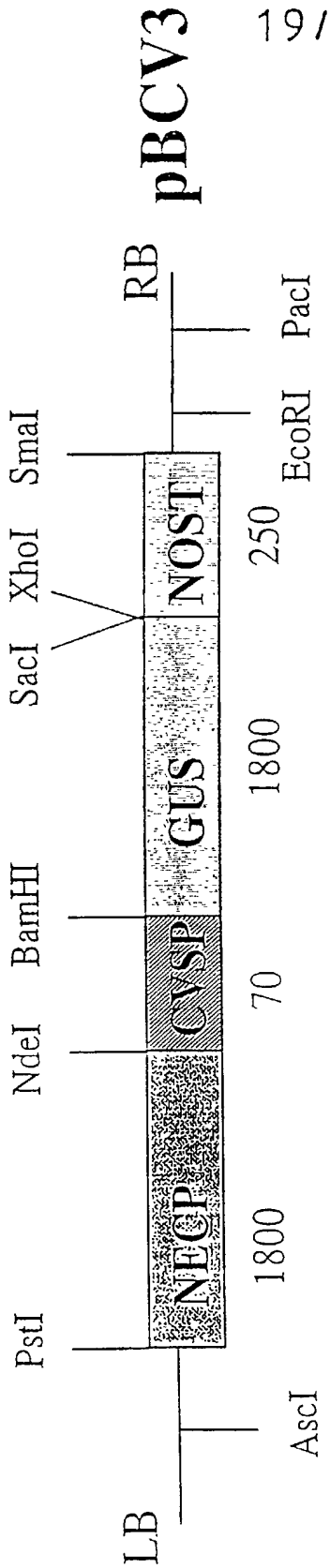
pBCV3
RB
PacI
EcoRI
SmaI
XhoI
SacI
BamHI
NdeI
PstI
LB
AscI
NOST
GUS
CVSP
NEGP
250
1800
70
1800

FIG. 18

```
1     TCTGAATACAAGCTGTGTGTGTAGAGAGATTTCATAAAGACAGCAAACAT
51    CCCTTCTTTTTGTTCTGTTTTAAAAGTTCCCTTCTTCAACCAGCTCTTTT
101   CCTCATCAGGGTAAGTTGCAAATAAAGGGGATGTTCCAGAATCAAGAAGA
151   GAAGATGTCAGACTCGCCTCAGAGGAAGATGGGAAGAGGAAAGATTGAGA
201   TTAAGAGGATTGAAAATACAACAAATCGTCAAGTCACTTTCTGTAAGAGA
251   AGAAATGGGTTGCTTAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGC
301   TGAAGTTGCTCTCATCGTTTTCTCAAGCCGTGGCCGCCTCTATGAATATG
351   CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA
401   GATTCCTCCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA
451   ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA
501   ACAGGAACATGCTAGGCGAGTCTCTAAGTTCTCTGACTGCAAAAGATCTG
551   AAAGGCCTGGAGACCAAACTTGAGAAAGGAATTAGTAGAATTAGGTCCAA
601   AAAGAATGAACTCCTGTTTGCTGAGATTGAGTATATGCGAAAAAGGGAAA
651   TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT
701   GAAAGAAATGTGAACATGATGGGAGGAGAATTTGAGCTGATGCAATCTCA
751   TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACAGCATAATC
801   ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTATAAGTTTAT
851   AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTTGGATTATG
901   TATAAGGGAGTGCAGGCCATTTGCCAATTATTGAAAGGTACTCAAACAGG
951   AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC
1001  TTAGCTTATGGACTCTAAAACAAAGACTTAATTTAACATATAAATATAAT
1051  TGTGTAATGCTGTTGTATTGTATGGTATGTATCCAAAAACATTAATAACC
1101  TATCTTTTTCTTCAAATTATGTCTCCTTTGATACAAACTACTAACATATT
1151  TTCTTAT
```

MADS-box

K-box

FIG.19

Practitioner's Docket No. _____ *PATENT*

Optional Customer No. Bar Code

00140

00140

PATENT TRADEMARK OFFICE

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION, OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type:

(check one applicable item below)

[ ] original.
[ ] design.

NOTE: With the exception of a supplemental oath or declaration submitted in a reissue, a supplemental oath or declaration is not treated as an amendment under 37 CFR 1.312 (Amendments after allowance) M P E.P. Section 714 16, 7th Ed

[ ] supplemental.

NOTE: If the declaration is for an International Application being filed as a divisional, continuation or continuation-in-part application, do not check next item; check appropriate one of last three items

[X] national stage of PCT.

NOTE. If one of the following 3 items apply, then complete and also attach ADDED PAGES FOR DIVISIONAL, CONTINUATION OR C-I-P.

NOTE: See 37 C.F.R. Section 1.63(d) (continued prosecution application) for use of a prior nonprovisional application declaration in the continuation or divisional application being filed on behalf of the same or fewer of the inventors named in the prior application

[ ] divisional.
[ ] continuation.

NOTE Where an application discloses and claims subject matter not disclosed in the prior application, or a continuation or divisional application names an inventor not named in the prior application, a continuation-in-part application must be filed under 37 C.F R. Section 1 53(b) (application filing requirements-nonprovisional application).

[ ] continuation-in-part (C-I-P).

INVENTORSHIP IDENTIFICATION

WARNING: *If the inventors are each not the inventors of all the claims, an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.*

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

PROCESS TO COLLECT METABOLITES FROM MODIFIED NECTAR BY INSECTS

SPECIFICATION IDENTIFICATION

The specification of which:

(complete (a), (b), or (c))

(a) [ ] is attached hereto.

NOTE: *"The following combinations of information supplied in an oath or declaration filed on the application filing date with a specification are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R. Section 1.63.*

"(1) name of inventor(s), and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration on filing;

"(2) name of inventor(s), and attorney docket number which was on the specification as filed; or

"(3) name of inventor(s), and title which was on the specification as filed "

Notice of July 13, 1995 (1177 O.G. 60)

(b) [ ] was filed on _____, [ ] as Application No. _____
[ ] and was amended on _____ (if applicable).

NOTE *Amendments filed after the original papers are deposited with the PTO that contain new matter are not accorded a filing date by being referred to in the declaration. Accordingly, the amendments involved are those filed with the application papers or, in the case of a supplemental declaration, are those amendments claiming matter not encompassed in the original statement of invention or claims. See 37 C.F.R. Section 1.67.*

NOTE. *"The following combinations of information supplied in an oath or declaration filed after the filing date are acceptable as minimums for identifying a specification and compliance with any one of the items below will be accepted as complying with the identification requirement of 37 C.F.R Section 1 63·*

(A) application number (consisting of the series code and the serial number, e.g., 08/123,456),
(B) serial number and filing date,
(C) attorney docket number which was on the specification as filed,
(D) title which was on the specification as filed and reference to an attached specification which is both attached to the oath or declaration at the time of execution and submitted with the oath or declaration; or
(E) title which was on the specification as filed and accompanied by a cover letter accurately identifying the application for which it was intended by either the application number (consisting of the series code and the serial number, e g , 08/123,456), or serial number and filing date Absent any statement(s) to the contrary, it will be presumed that the application filed in the PTO is the application which the inventor(s) executed by signing the oath or declaration.
M.P.E P. Section 601 01(a), 7th ed

(c) [X] was described and claimed in PCT International Application No. PCT/NL99/00453 filed on 15 July 1999 and as amended under PCT Article 19 on _____*(if any)*.

SUPPLEMENTAL DECLARATION (37 C.F.R. Section 1.67(b))

(complete the following where a supplemental declaration is being submitted)

[ ] I hereby declare that the subject matter of the

[ ] attached amendment
[ ] amendment filed on _____.

was part of my/our invention and was invented before the filing date of the original application, above identified, for such invention.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, Section 1.56,

(also check the following items, if desired)

[ ] and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, and

[ ] in compliance with this duty, there is attached an information disclosure statement, in accordance with 37 C.F.R. Section 1.98.

PRIORITY CLAIM (35 U.S.C. Section 119(a)-(d))

NOTE. "The claim to priority need be in no special form and may be made by the attorney or agent if the foreign application is referred to in the oath or declaration as required by Section 1 63. The claim for priority and the certified copy of the foreign application specified in 35 U.S C. Section 119(b) must be filed in the case of an interference (Section 1.630), when necessary to overcome the date of a reference relied upon by the examiner, when specifically required by the examiner, and in all other situations, before the patent is granted. If the claim for priority or the certified copy of the foreign application is filed after the date the issue fee is paid, it must be accompanied by a petition requesting entry and by the fee set forth in Section 1.17(i). If the certified copy is not in the English language, a translation need not be filed except in the case of interference, or when necessary to overcome the date of a reference relied upon by the examiner, or when specifically required by the examiner, in which event an English language translation must be filed together with a statement that the translation of the certified copy is accurate." 37 C F R. Section 1.55(a)

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [X] such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S itself claimed priority check item (e), enter the details below and make the priority claim

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. SECTION 119(a)-(d)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING DAY, MONTH, YEAR	PRIORITY CLAIMED UNDER 35 USC 119
Europe	98202375.6	16 July 1998	[X]YES [ ]NO
Europe	98204215.2	14 December 1998	[X]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO
			[ ]YES [ ]NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. Section 119(e))

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER **FILING DATE**

/ _____ _____
/ _____ _____
/ _____ _____

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. SECTION 120

[ ] The claim for the benefit of any such applications are set forth in the attached ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART (C-I-P) APPLICATION.

ALL FOREIGN APPLICATION(S), *IF ANY,* FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

NOTE: If the application filed more than 12 months from the filing date of this application is a PCT filing forming the basis for this application entering the United States as (1) the national stage, or (2) a continuation, divisional, or continuation-in-part, then also complete ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR C-I-P APPLICATION for benefit of the prior U S. or PCT application(s) under 35 U.S.C. Section 120

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

(list name and registration number)

12

JOSEPH H. HANDELMAN, 26179
JOHN RICHARDS, 31053
RICHARD J. STREIT, 25765
PETER D. GALLOWAY, 27885
IAN C. BAILLIE, 24090
THOMAS F. PETERSON, 24790
RICHARD P. BERG, 28145
JULIAN H. COHEN, 20302
WILLIAM R. EVANS 25858
JANET I. CORD, 33778
CLIFFORD J. MASS, 30086
CYNTHIA R. MILLER, 34678

(Check the following item, if applicable)

[ ] I hereby appoint the practitioner(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

[ ] Attached, as part of this declaration and power of attorney, is the authorization of the above-named practitioner(s) to accept and follow instructions from my representative(s).

NOTE: "Special care should be taken in continuation or divisional applications to ensure that any change of correspondence address in a prior application is reflected in the continuation or divisional application. For example, where a copy of the oath or declaration from the prior application is submitted for a continuation or divisional application filed under 37 CFR 1.53(b) and the copy of the oath or declaration from the prior application designates an old correspondence address, the Office may not recognize, in the continuation or divisional application, the change of correspondence address made during the prosecution of the prior application Applicant is required to identify the change of correspondence address in the continuation or divisional application to ensure that communications from the Office are mailed to the current correspondence address. 37 CFR 1 63(d)(4) " Section 601.03, M.P E.P , 7th Ed

SEND CORRESPONDENCE TO

Ladas & Parry
26 West 61st Street
New York, N.Y. 10023

DIRECT TELEPHONE CALLS TO:
(Name and telephone number)

(complete the following if applicable)

Since this filing is a [ ] continuation [ ] divisional there is attached hereto a Change of Correspondence Address so that there will be no question as to where the PTO should direct all correspondence.

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

NOTE: Carefully indicate the family (or last) name, as it should appear on the filing receipt and all other document.

NOTE. Each inventor must be identified by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and by his/her residence, post office address and country of citizenship. 37 C F.R Section 1.63(a)(3).

NOTE Inventors may execute separate declarations/oaths provided each declaration/oath sets forth all the inventors. Section 1.63(a)(3) requires that a declaration/oath, inter alia, identify each inventor and prohibits the execution of separate declarations/oaths which each sets forth only the name of the executing inventor. 62 Fed Reg 53,131, 53,142, October 10, 1997,

Full name of sole or first inventor
Jantina | (Middle Initial or Name) | CREEMERS
(Given Name) *(Middle Initial or Name)* ***Family (Or Last Name)***

100

Inventor's signature _____

Date JAN 2 9 2001 **Country of Citizenship** Netherlands

Residence c/o Stichting Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), Droevendaalsesteeg 1, 6708 PB WAGENINGEN, The Netherlands

Post Office Address same as above NLX

,,,,,,

Full name of second joint inventor, if any
Gerrit | Cornelis | ANGENENT
(Given Name) *(Middle Initial or Name)* ***Family (Or Last Name)***

200

Inventor's signature _____

Date JAN 2 9 2001 **Country of Citizenship** Netherlands

Residence c/o Stichting Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), Droevendaalsesteeg 1, 6708 PB WAGENINGEN, The Netherlands

Post Office Address same as above NLX

,,,,,,

Full name of third joint inventor, if any
Martin | Maria | KATER
(Given Name) *(Middle Initial or Name)* ***Family (Or Last Name)***

300

Inventor's signature _____

Date JAN 2 5 2001 **Country of Citizenship** Netherlands

Residence c/o Stuchting Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), Droevendaalsesteeg 1, 6708 PB WAGENINGEN, The Netherlands

Post Office Address same as above NLX

(check proper box(es) for any of the following added page(s) that form a part of this declaration)

[ ] **Signature** for fourth and subsequent joint inventors. *Number of pages added* _____

* * *

[ ] **Signature** by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. *Number of pages added* _____

* * *

[ ] **Signature** for inventor who refuses to sign or cannot be reached by person authorized under 37 C.F.R. Section 1.47. *Number of pages added* _____

* * *

[ ] Added page for **signature** by one joint inventor on behalf of deceased inventor(s) where legal representative cannot be appointed in time. (37 C.F.R. Section 1.47)

* * *

[ ] Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (C-I-P) application.

[ ] Number of pages added _____

* * *

[ ] Authorization of practitioner(s) to accept and follow instructions from representative.

(If no further pages form a part of this Declaration, then end this Declaration with this page and check the following item)

[X] This declaration ends with this page.

SEQUENCE LISTING

<110> CREEMERS, Jantina
ANGENENT, Gerrit
KATER, Martin

<120> Process to collect metabolites from modified nectar by insects

<130> U-13212-4

<140> 09/743885
<141> 2001-01-16

<160> 29

<170> PatentIn Ver. 2.1

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<211> 265
<212> PRT
<213> Petunia x hybrida

<220>
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<220>
<223> tissue type: nectar gland

<220>
<223> NEC1 amino acid sequence

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Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala Pro Val Pro Thr Phe
             20                  25                  30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
         35                  40                  45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
     50                  55                  60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
 65                  70                  75                  80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
                 85                  90                  95

Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
            100                 105                 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
        115                 120                 125
```

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Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
    130                 135                 140

Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
145                 150                 155                 160

Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
                165                 170                 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
            180                 185                 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
        195                 200                 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
    210                 215                 220

Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
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Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
             20                  25                  30

Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
         35                  40                  45

Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
     50                  55                  60

Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
 65                  70                  75                  80
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Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
85 90 95

Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
100 105 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
115 120 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
130 135 140

Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145 150 155 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
165 170 175

Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
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Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
195 200 205

Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
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His Ala

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cttcttggta atattgtatc attcatggtc ttcctagcac ccgtgccaac attttacaaa 180
atatataaaa ggaaatcatc agaaggatat caagcaatac catatatggt agcactgttc 240
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<220>
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flowers

<220>
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<220>
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<220>
<223> Description of Artificial Sequence: primer prat
122

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<210> 9
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<210> 10
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<213> Artificial Sequence

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<212> DNA
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<210> 12
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<220>
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149

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<210> 13
<211> 35
<212> DNA
<213> Artificial Sequence

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169

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<210> 14
<211> 32
<212> PRT
<213> Calluna vulgaris

<220>
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1 5 10 15

Ala Gly Tyr Ser Cys Thr Glu Pro Ser Thr Val Thr Ser Gln Asp Phe
20 25 30

<210> 15

<211> 40
<212> PRT
<213> Calluna vulgaris

<220>
<223> CVH 29 N-terminal sequence

<400> 15
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1 5 10 15

Ala Gly Tyr Ser Cys Lys Glu Pro Ala Lys Val Thr Val Asp Asp Phe
20 25 30

Val Phe His Gly Leu Gly Thr Ala
35 40

<210> 16
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<210> 17
<211> 20
<212> DNA
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<210> 19
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<211> 28
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<220>
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248

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cgccatatgt ttttttatgg aagcccc 27

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<220>
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tcttgatttc 70

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attggaagga aca 73

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<220>
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250

<400> 27

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<210> 29
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<212> DNA
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<220>
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aaatgtcttt gatgcataat atttcccata atc 33

SEQUENCE LISTING

<110> CPRO-DLO

<120> Process to collect metabolites from modified nectar by insects

<130> 159782

<140> pct/nl99/00453
<141> 1999-07-15

<160> 10

<170> PatentIn Ver. 2.1

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<213> Petunia x hybrida

<220>
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<220>
<223> tissue type: nectar gland

<220>
<223> NEC1 amino acid sequence

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            20                  25                  30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
        35                  40                  45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
    50                  55                  60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
65                  70                  75                  80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
                85                  90                  95
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Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
            100                 105                 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
        115                 120                 125

Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
    130                 135                 140

Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
145                 150                 155                 160

Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
                165                 170                 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
            180                 185                 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
        195                 200                 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
    210                 215                 220

Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
225                 230                 235                 240

Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu Gln Ser Met Glu Lys
                245                 250                 255

Asp Phe Ser Arg Leu Arg Thr Ser Lys
            260                 265
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<210> 2
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<212> PRT
<213> Petunia x hybrida

<220>
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<220>
<223> tissue type: nectar gland, secretory cell

<220>

<223> FBP15 amino acid sequence

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             20                  25                  30

Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
         35                  40                  45

Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
     50                  55                  60

Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
 65                  70                  75                  80

Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
                 85                  90                  95

Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
            100                 105                 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
        115                 120                 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
    130                 135                 140

Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145                 150                 155                 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
                165                 170                 175

Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
            180                 185                 190

Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
        195                 200                 205

Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
    210                 215                 220
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<211> 18
<212> PRT
<213> Calluna vulgaris

<220>
<223> tissue type: flower

<220>
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His Ala

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<212> DNA
<213> Petunia x hybrida

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<222> (79)..(873)

<220>
<223> strain: W115

<220>
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cttcactaaa aaaaaatc atg gcg caa tta cgt gct gat gac ttg tct ttc 111
Met Ala Gln Leu Arg Ala Asp Asp Leu Ser Phe
1 5 10

ata ttt ggc ctt ctt ggt aat att gta tca ttc atg gtc ttc cta gca 159
Ile Phe Gly Leu Leu Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala
15 20 25

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ccc gtg cca aca ttt tac aaa ata tat aaa agg aaa tca tca gaa gga   207
Pro Val Pro Thr Phe Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly
          30                  35                  40

tat caa gca ata cca tat atg gta gca ctg ttc agc gcc gga cta ttg   255
Tyr Gln Ala Ile Pro Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu
      45                  50                  55

cta tat tat gct tat ctc agg aag aat gcc tat ctt atc gtc agc att   303
Leu Tyr Tyr Ala Tyr Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile
  60                  65                  70                  75

aat ggc ttt gga tgt gcc att gaa tta aca tat atc tct ctg ttt ctc   351
Asn Gly Phe Gly Cys Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu
                  80                  85                  90

ttt tac gcg ccc aga aag tct aag att ttc aca ggg tgg ctg atg ctc   399
Phe Tyr Ala Pro Arg Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu
              95                 100                 105

tta gaa ttg gga gcc cta gga atg gtg atg cca att act tat tta tta   447
Leu Glu Leu Gly Ala Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu
         110                 115                 120

gca gaa ggc tca cat aga gtg atg ata gtg gga tgg att tgt gca gct   495
Ala Glu Gly Ser His Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala
     125                 130                 135

atc aat gtt gct gtc ttt gct gct cct tta agc atc atg agg caa gta   543
Ile Asn Val Ala Val Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val
140                 145                 150                 155

ata aaa aca aag agt gta gag ttc atg ccc ttc act tta tct ttg ttc   591
Ile Lys Thr Lys Ser Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe
                160                 165                 170

ctc act ctc tgt gcc act atg tgg ttt ttc tat ggg ttt ttc aag aag   639
Leu Thr Leu Cys Ala Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys
            175                 180                 185

gac ttt tac att gcg ttt cca aat ata ctg ggc ttt cta ttc gga atc   687
Asp Phe Tyr Ile Ala Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile
        190                 195                 200

gtt caa atg cta tta tat ttt gtt tac aag gat tca aag aga ata gat   735
Val Gln Met Leu Leu Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp
    205                 210                 215
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gat gaa aaa tct gat cct gtt cga gaa gct aca aaa tca aaa gaa ggt   783
Asp Glu Lys Ser Asp Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly
220                 225                 230                 235

gta gaa atc att atc aac att gaa gat gat aat tct gat aac gca ttg   831
Val Glu Ile Ile Ile Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu
                240                 245                 250

cag tcc atg gag aag gat ttt tcc aga ctg cgg aca tca aaa           873
Gln Ser Met Glu Lys Asp Phe Ser Arg Leu Arg Thr Ser Lys
            255                 260                 265

taagcaagaa gatgatcaaa aaatgacaaa gctaaggagt ttgaagtaag gcaaggaact 933

tgacactgaa tatctaagct aattagcaag actttagcag cttgtaatat ttagtgtttg 993

tgaggtgtta ccttataatt agcttgtagc atagccttcc cactaataat tctgcttagc 1053

gaatcttata tatgggaaat acttacacta gtatgcatct tctatataca tgtttggcac 1113

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<212> PRT
<213> Petunia x hybrida
<223> NEC1

<400> 5

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Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala Pro Val Pro Thr Phe
             20                  25                  30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
         35                  40                  45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
     50                  55                  60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
 65                  70                  75                  80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
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                85                  90                  95

Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
            100                 105                 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
        115                 120                 125

Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
    130                 135                 140

Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
145                 150                 155                 160

Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
                165                 170                 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
            180                 185                 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
        195                 200                 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
    210                 215                 220

Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
225                 230                 235                 240

Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu Gln Ser Met Glu Lys
                245                 250                 255

Asp Phe Ser Arg Leu Arg Thr Ser Lys
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<211> 1157
<212> DNA
<213> Petunia x hybrida

<220>
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<222> (179)..(841)

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<223> strain: W115

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<223> tissue type: nectar gland

<220>
<223> cDNA library of nectaries from Petunia hybrida
flowers

<220>
<223> FBP15

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aataaagggg atgttccaga atcaagaaga gaagatgtca gactcgcctc agaggaag 178

atg gga aga gga aag att gag att aag agg att gaa aat aca aca aat 226
Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn
1 5 10 15

cgt caa gtc act ttc tgt aag aga aga aat ggg ttg ctt aaa aaa gct 274
Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
20 25 30

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Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
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Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
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Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
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Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
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Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu

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Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
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His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
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Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
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Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
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His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
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Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
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Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
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